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The DNA replication factor RPA physically associates with the tumor suppressor protein p53, an interaction that could be important for the function of both these proteins in normal and cancer cells. Using two mutant forms of p53 with the desired property of not binding RPA we have demonstrated that RPA binding is not required for growth suppression by p53. We have also made a deletion mutant of RPA which does not bind p53 to show that despite binding single-stranded DNA, this mutant of RPA fails to support DNA replication. Therefore the region of RPA which interacts with p53 is essential for the protein complex's activity. In contrast to RPA binding, transcription trans-activation by p53 is essential for growth suppression. One of targets of p53, p21/WAF1/CIP1, inhibits the cell-cycle by associating with cyclin-cdk kinases. It also inhibits DNA replication by interacting with a replication factor, Proliferating Cell Nuclear Antigen (PCNA). We have characterized a 39 amino acid fragment of p21 which can target PCNA in vitro and in vivo and suggest that small chemicals based on the structure of this peptide could be useful for targeting the DNA replication apparatus for chemotherapy of breast cancers.

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This work is being done in concert with a postdoctoral fellow in my laboratory, Dr. Junjie Chen, who is independently supported by a postdoctoral fellowship: DAMD17-94-J-4070. He is writing a separate report. Although there is overlap in the work done because it is being done in concert by a postdoctoral fellow (Dr. Junjie Chen) and a principal investigator (Dr. Anindya Dutta), we have written separate reports dealing with separate aspects of the work. The grants support our separate salaries. Publications resulting from this work are listed below and reprints are included

Leiter L M, Chen J, Marathe T, Tanaka M and Dutta A. Loss of transactivation and transrepression function, and not RPA binding, alters growth suppression by p53. Oncogene. 1996; 12, 2661-2668.

Lin Y-L, Chen C, Keshav K F, Winchester E and Dutta A. Dissection of functional domains of the human DNA replication protein complex Replication Protein A. J. Biol. Chemistry. 1996; 271, 17190-17198.

Chen J, Peters R, Saha P, Lee P, Theodoras A, Pagano M, Wagner G and Dutta A. A 39 amino acid fragment of the cell cycle regulator p21 is sufficient to bind PCNA and partially inhibit DNA replication in vivo. Nucleic Acids Research. 1996; 24, 1727-1733.

INTRODUCTION

In the report for 1995, I highlighted the importance of p53 function in breast cancer, given a short introduction to p53 and RPA, and introduced how we believed that the function of RPA may be regulated by p53. Briefly, p53, a tumor suppressor mutated in up to 50% of breast cancers, is a transcriptional activator and independently interacts with the DNA replication factor RPA. We had shown that by interacting with RPA, p53 inhibits one of the major functions of RPA, binding to single-stranded DNA. The aim of the project was to determine the significance of the RPA-p53 interaction for growth suppression by p53.

We aimed to do this by selectively mutating p53 such that it no longer binds RPA and yet retains transcription activation. In the 1995 annual report I reported that we had p53 molecules with certain point mutations, W53S-F54S (where the tryptophan at position 53 and the phenylalanine at position 54 were changed to serine residues) and D48H-D49H (aspartic acid at positions 48 and 49 changed to histidine), which had lost the ability to bind RPA but retained the ability to activate transcription. In this year we determined the effects of these mutations on growth suppression by p53. The result have been published (1).

We also embarked on making mutations in Rpa1, the largest subunit of RPA which is responsible for binding p53 and to single-stranded DNA, with the aim of making a mutant form of RPA which did not bind to p53. We have completed this goal in this year and the effects of the mutations on RPA function have been published (2).

A change in the direction of research has become necessary based on the results published in (1). The results strongly suggest that the main growth suppressive action of p53 derives from its ability to activate transcription rather than binding to and inhibiting RPA. Since the fundamental rationale of this project is to restore to breast cancer cells growth restraints normally exerted by wild type p53, our result suggest that we should not restrict our research to studying the RPA-p53 interaction. One of the major effectors of p53 has been discovered in the last few years, a protein variously called p21/WAF1/CIP1. p53 induces the mRNA for this gene, p21 protein product increases and it suppresses cell growth. Indeed, "knock-out" mice with homozygous deletions of the p21 gene lose one of the important effects of p53: despite having wild type alleles of p53 they fail to stop at the G1-S transition following radiation (3, 4). This is an

important loss because the G1-S block prevents the cell from replicating its DNA before it has had time to repair DNA damage. As a result of this failure to stop at G1-S, DNA-damage induced mutations are propagated to the progeny cells. Acquisition of new mutations is now recognized as a hallmark of cancer development and progression. Thus the function of p21 is crucial for normal growth control by p53 and restoring p21 function in p53 mutant cancers could have a therapeutic advantage. Of course, this also means that we need to understand how p21 stops cell growth at the G1-S transition.

The cell-cycle: As a cell proliferates, it passes through well-defined phases named G1 (preparation for DNA replication), S (replication of its DNA), G2 (preparation for mitosis) and M (mitosis and cytokinesis) in a cyclical manner called the cell-cycle. The cell-cycle engine is driven by the periodic expression of regulatory proteins, cyclins, which associate with and activate the kinase activities of catalytic subunits called cyclin-dependent kinases (cdks), and which are destroyed as the cell progresses through the relevant phase of the cell-cycle. For example, the G1 cyclin, Cyclin E, is expressed in G1, associates with and activates various cdks, particularly cdk2, and is degraded as the cells enter S phase (5-7). Cyclin A, on the other hand, appears in S phase, associates with cdk2 and cdc2, and is degraded in M. Both cyclin E and/or cyclin A promote the entry of cells into S. Post-translational modifications on the cdks are also necessary to activate the kinase activities. Recently a third mode of regulation of the cyclin-cdk kinases has been discovered, the cdk inhibitors, which associate with and inactivate the cyclin-cdk kinases (8).

p21/CIP1/WAF1: p21, a gene which is induced by p53, codes for a 21 kD protein which associates with and inhibits cyclin-cdk kinases (9-13) . Beside inhibiting cyclin-cdk kinases, the p21 protein directly interacts with and inhibits an essential DNA replication factor, proliferating cell nuclear antigen (PCNA) (14, 15). In a recent paper (16) we showed that the regions of p21 involved in interacting with and inhibiting (i) the cyclin - cdk kinases and (ii) PCNA are separable from each other. The N terminal domain of p21 (p21N) interacts with the cdk2 protein and inhibits cyclin-cdk kinase activity, while the C terminal domain (p21C) interacts with and inhibits PCNA. Using these separated domains we showed that p21N inhibits DNA replication in Xenopus egg extracts, and inhibits growth of transformed human osteosarcoma cells, SaOs2, while p21C inhibits the SV40 based DNA replication reaction. These results suggest that the minimal requirement for growth suppression by p21 is its ability to inhibit the cyclin-cdk kinases. Dr. Junjie Chen will describe in his report how we have followed the exact mechanism by which p21 inhibits cyclin-cdk kinases. Briefly we have discovered that p21 uses two separate motifs, a cyclin binding Cy motif and a cdk binding K motif which independently bind to the cyclin and the cdk, with both these motifs being essential for optimal kinase inhibition and cell growth suppression.

In my report, I have focused on the PCNA inhibiting activity of p21. The discovery that one of the effectors of p53 has an independent action on the DNA replication apparatus (through PCNA) is similar to our original observation that p53 has an independent action on the DNA replication machinery (through RPA). Since the results reported below suggest that RPA-p53 interaction is not important, but production of p21 is important, for growth-suppression by p53 we have pursued the p21-PCNA interaction as a new task in the second year of this project.

PCNA: PCNA is an auxiliary factor for DNA polymerases delta and epsilon and is essential for DNA replication *in vitro* and *in vivo* (17, 18). p21 interacts with PCNA and inhibits its activity (14, 15) . PCNA promotes the processivity of DNA polymerase delta allowing it to synthesize long strands of DNA necessary for replicating the leading strand. PCNA has a ring shaped structure made up of three subunits which assembles around DNA like a "ring around a curtain-rod". The structure suggests that its mechanism of action is to move along the DNA like a sliding clamp to which the polymerase delta is tethered (19).

BODY

SPECIFIC AIMS FOR YEAR 2

1. Determine the effects of mutations in p53 that abolish binding to RPA on growth suppression by p53. (Task 2b).
2. Mutate the part of Rpa1 that binds p53 and determine effect on RPA function. (Task 3, second half).
3. Determine whether a peptide derived from the sequence of p21, one of the effectors of p53, inhibits DNA replication in vitro and in vivo. (New Task , based on the revisions discussed in the introduction).

METHODS

Growth suppression by stable transfections. CMV/p53 mutants L14Q-F19S, L22Q-W23S, D48H-D49H and D61H-E62K were the kind gift of Dr. Arnold Levine (mutations described in (1)). p53 wild type and W53S-F54S mutants were cloned into a mammalian expression vector cDNA3 (Invitrogen) . These plasmids were transfected into SaOs2, a human osteosarcoma cell line with loss of both alleles of p53, as well as H1299, a human lung large cell carcinoma cell line with partial homozygous deletion of the p53 gene, by the calcium phosphate method. The ability of each plasmid to produce G418 resistant colonies was measured as described (16).
RPA mutations: The plasmids for expressing RPA holocomplex with deletions or mutations in Rpa1 were derived from p11dtRPA provided by Dr. Marc Wold which expressed wild-type human RPA in bacteria (2). pm11dtRPA, p11dtRPAΔ222-411 and p3atRPA278-616 express wild type Rpa2 and 3 but Rpa1 with a mutation in the zinc finger, a deletion of amino acids 222-411 or a deletion of amino acids 1-277, respectively. Details of their construction and purification have been published (2). The protein complexes were named according to the Rpa1 mutant present in the complex.
Association of recombinant RPA with GST-p53: this was measured essentially as described in last year's report, the only difference being that bound RPA was detected by SDS-PAGE followed by immunoblotting with anti-Rpa1 and anti-Rpa2 antibodies.
Synthesis of p21 based and control peptides and proteins

pGST-p21, -p21N and -p21C were generated as described (16). pGST-p21M1 and -p21C2 were generated by PCR with Pfu polymerase and cloned into BamHI and SalI sites of pGEX-5X3 (Promega) . Bacterially produced proteins were expressed in E. coli BL21. Protein induction, cell lysis and affinity-purification with glutathione-agarose beads were done as described (16). A 41 amino acid p21C2 peptide (consisting of the 39 C-terminal amino acids of p21 plus two lysine residues at the carboxy-terminal end required for chemical synthesis) was synthesized and purified using C18 reverse phase HPLC.

The sequences of peptides used were:
p21C2: QAEGSPGGPGDSQGRKRRQTSMTDFYHSKRRLIFSKRKPKK
CSH262:WNSGFESYGSSSYGGAGGYTQAPGGFGAPAPSQAEKKSRAR
CSH119: ADAQHAAPPKKKRKVEDPKDF
SV40 based DNA replication reaction. Replication of a plasmid containing SV40 origin of DNA replication was carried out in human cell extracts depleted of RPA (1), which have been supplemented with the bacterially expressed RPA holocomplexes.
Microinjection. IMR90 human diploid fibroblast monolayers growing on glass coverslips (at 60% density) were synchronized in G0 by serum starvation for 48 hr. and stimulated to enter G1 by addition of 10% fetal bovine serum. 15 hr after re-activation, cells in G1 were microinjected with rabbit immunoglobulin (IgG) as marker and the indicated proteins using an automated microinjection system (AIS, Zeiss) (20).

DNA synthesis was monitored by incubating with BrdU (100 μM, Amersham) for 10-12 hr after microinjection. Micro-injected cells were detected by incubation for one hour with biotinylated horse anti-rabbit IgG (Vector Laboratories, dilution 1:50), and stained with mouse monoclonal anti-BrdU antibody plus an anti-mouse FITC-conjugated antibody (Vector Laboratories). In each experiment about 100 injected cells (and corresponding number of non-injected cells) were counted. % of Inhibition of BrdU incorporation was calculated as {(N-I)/N} x 100, where N is the percentage of BrdU incorporation in non-injected cells and I is the percentage of BrdU in cells microinjected with antibodies.

RESULTS

Growth suppression by stable transfection: Plasmids expressing wild type or mutant p53 were transfected into SaOs2 and H1299 cells (deficient in endogenous p53) and G418 resistant colonies selected (Fig. 1). As demonstrated by others, plasmids expressing wild type p53 established very few G418 resistant colonies compared to the vector which does not express p53, due to growth suppression by p53. The results from the other plasmids demonstrate that both p53 proteins with wild type transcription trans-activation but diminished RPA binding, D48H-D49H and W53S-F54S, exhibited as much growth suppression as wild type p53 proteins. Therefore, both forms of p53 with diminished RPA binding retained growth suppression.

The p53 protein L22Q-W23S, which had wild type RPA binding activity but reduced transcription trans-activation showed diminished growth suppression in both SaOs2 and H1299 cells. The L14Q-F19S and D61H-E62K mutants, which retained most of the trans-activation functions, also retained most of the growth suppression activity of wild-type p53 in both SaOs2 and H1299 cells. These results imply the trans-activation by p53 is important for growth suppression.

Region of Rpa1 required for binding p53. We confirmed and extended the results using purified RPA holocomplexes with selected deletion derivatives of Rpa1 (Fig. 2a) and analyzed for binding to p53 (Fig. 2b). RPA with Δ222-411 Rpa1 bound to p53 indicating that the middle third of Rpa1 was not required for this activity. The failure of RPA with 278-616 Rpa1 to bind p53 confirms that the N terminal 1-278 amino acids of Rpa1 are essential for the interaction. However the 1-278 region of Rpa1 alone was unable to bind to p53 (2). Taken together, we conclude that the N terminal 221 amino acids of Rpa1 together with residues in the 411-492 region are sufficient for binding p53.

The inability of mutant RPA which does not bind to p53 to support DNA replication: The RPA holocomplexes were tested for their ability to support SV40 based DNA replication in an extract depleted of endogenous RPA. None of the mutant forms of RPA supported DNA replication (Fig. 2c). 278-616 RPA bound single-stranded DNA to an extent comparable with that of wild type RPA but did not support any DNA replication. This result suggests that a deletion in Rpa1 which affects binding to p53 simultaneously causes Rpa1 to become ineffective for DNA replication, despite the fact that ssDNA binding is unaffected.

Inhibition of the SV40 based in vitro DNA replication reaction by a peptide derived from p21.

Since the interaction of p21 with PCNA inactivates its function as a DNA replication factor, we measured the abilities of the GST fusion proteins to inhibit the SV40 based DNA replication reaction (Fig. 3). The concentration required to obtain 50% inhibition of replication (IC50) was 0.5 to 1 nM for GST-p21 or GST-p21C and 9 nM for GST-p21C2. The synthetic p21C2 peptide was slightly weaker than GST-p21C2 at inhibiting SV40 replication (IC50 = 14 nM), but addition of 1% DMSO to the replication reaction improved inhibition by the p21C2 peptide about 2 fold (data not shown). The 10-20 fold weaker inhibitory activity of GST-p21C2 compared to GST-p21C could be

consistent with its lower affinity for PCNA at 37 °C. The inhibition of DNA replication by p21C2 was reversed by the addition of excess PCNA (data not shown). We tested whether amino acids 87-125 of p21 (present in p21C but not in p21C2) contributed to the inhibition of SV40 DNA replication by interacting with and inhibiting a second replication factor. A fragment of p21 containing this region, GST-p21M1, was unable to bind PCNA or inhibit the DNA replication reaction (Fig. 3). These results suggest that amino acids 87-125 of p21 contribute to replication inhibition only by stabilizing the p21-PCNA interaction. However the 39 amino acid peptide of p21 was still an effective inhibitor of DNA replication in vitro.

Effect of GST-p21C and p21C2 peptide on entry of quiescent cells into S phase.

To determine whether a p21 based peptide was active in vivo at reaching and interacting with PCNA, we analyzed whether S phase was inhibited by these proteins. Quiescent diploid fibroblasts were stimulated by serum and entry into S phase followed after micro-injection of GST-fusion proteins or the p21C2 peptide (Fig. 4). GST-p21 - p21N and -p21C inhibited uptake of Bromodeoxyuridine significantly compared to a negative control peptide CSH119, GST alone, or GST fused to a cell-cycle regulatory protein cdc25C (21). Thus, GST-p21C inhibits growth of cells almost as well as GST-p21N when provided in high enough concentrations. Consistent with the result from the in vitro SV40 replication reaction, GST-p21C2 inhibited entry into S phase although less effectively than GST-p21C. Surprisingly, the p21C2 peptide was only a weak inhibitor of cell growth. The difference between GST-p21C2 and the p21C2 peptide was observed consistently and was statistically significant ($p < 0.05$ in ANOVA). The results also confirm earlier reports that p21N, which binds and inhibits cdk kinases but not PCNA, inhibits growth of cells almost as effectively as p21.

FIGURE LEGENDS

Fig. 1 Growth suppression of wild type p53 and mutants in stable transfection assays. Bars represent the mean (± standard error of the mean) of the number of colonies for 14 (SaOs2) and 4-5 transfections (H1299) compared to cDNA3 (= 100%, no growth suppression). Data were analyzed by one-way ANOVA and means were categorized by Fisher's LSD test. * indicates a significant difference compared to all other p53 alleles at $p < 0.0003$ (SaOs2) and $p < 0.0001$ (H1299). The p53 proteins were wt (wild-type); 14,19 (L14Q-F19S); 22,23 (L22Q-W23S); 48,49 (D48H-D49H); 53,54 (W53S-F54S) and 61,62 (D61H-E62K).

Fig. 2 RPA holocomplexes prepared with selected mutants of the largest subunit, Rpa1, were tested for their p53 binding and DNA replication activities. (a) Coomasie stain of 2 μg of each RPA holocomplex containing Rpa2, Rpa3 and indicated versions of Rpa1 obtained by expression in E. coli followed by purification as described in (2). 1-616: wild type RPA, m1-616 RPA: Rpa1 has a point mutation that removes the evolutionarily conserved zinc finger. 278-616 RPA: Rpa1 has a deletion of amino acids 1-277. Δ222-411 RPA: Rpa1 has a deletion of amino acids 222-411. (b) The RPA holocomplexes purified were tested for their ability to bind GST-p53. The Rpa1 and Rpa2 proteins present in the indicated lanes were visualized by immunoblotting with monoclonal antibodies mAb p70-9 (αRpa1) and mAb p34-20 (αRpa2) respectively. 0.1 x input : one-tenth of protein input into reaction. GST: protein bound by GST beads. GST-p53: protein bound by GST-p53 beads. (c) DNA replication was studied in an SV40 based in vitro reaction using T antigen, 293 S100 cell extract selectively depleted of endogenous RPA and indicated amounts of recombinant RPA complexes in a 25 μl reaction. pmoles of α^{32}P dAMP incorporated into polynucleotide (in a theoretical 50 μl reaction) measures

extent of DNA replication. The RPA holocomplexes are: 1-616 RPA (open circles), m1-616 RPA (crosses), 278-616 RPA (open squares) and Δ222-411 RPA (open triangles).

Fig. 3 Inhibition of SV40 DNA replication by fragments of p21. The proteins added were GST-p21 (open squares), GST-p21C (open circles), GST-p21C2 (closed circles), GST-p21M1 (open triangles) and p21C2 peptide (closed squares). Each point represents the mean and standard deviation of three separate measurements of DNA replication (amount of dAMP incorporated into polynucleotide).

Fig. 4 Inhibition of entry into S phase by microinjection of GST-p21 fusion proteins and indicated peptides into nuclei of serum re-activated diploid fibroblasts 15 hr after re-activation. Mean and standard deviation for at least three different experiments are shown. CSH119 is the negative control, with indicated growth inhibition probably being a side-effect of the injection procedure.

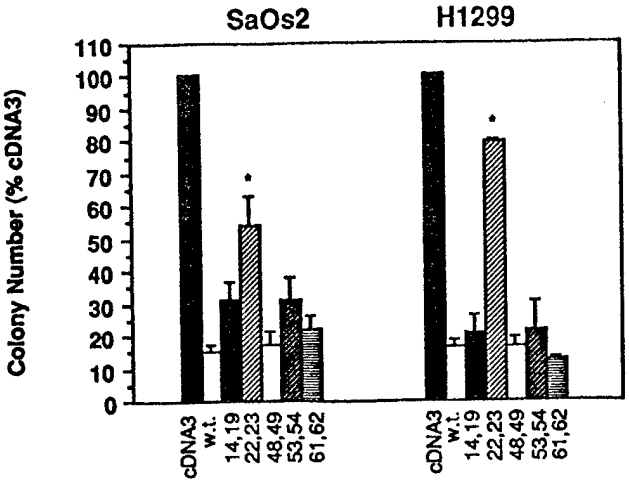

Figure 1

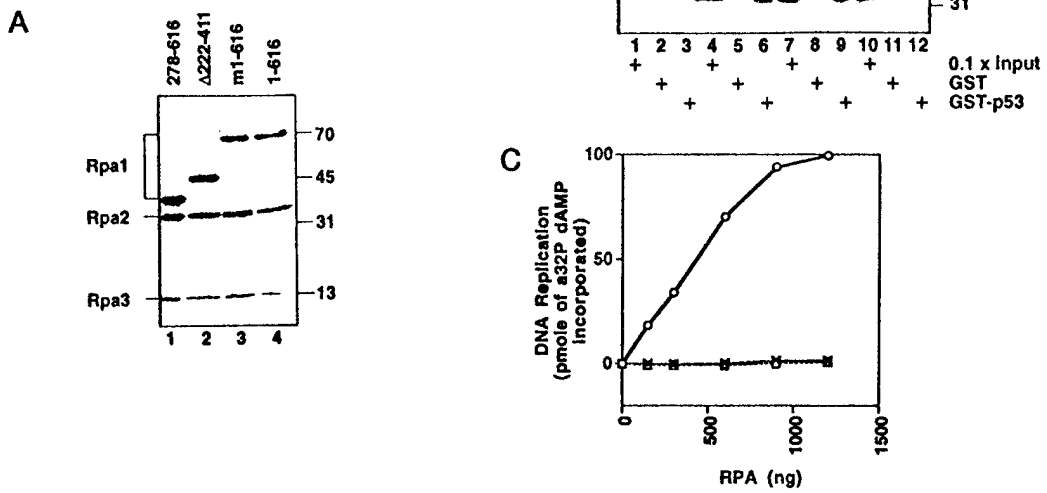

Figure 2

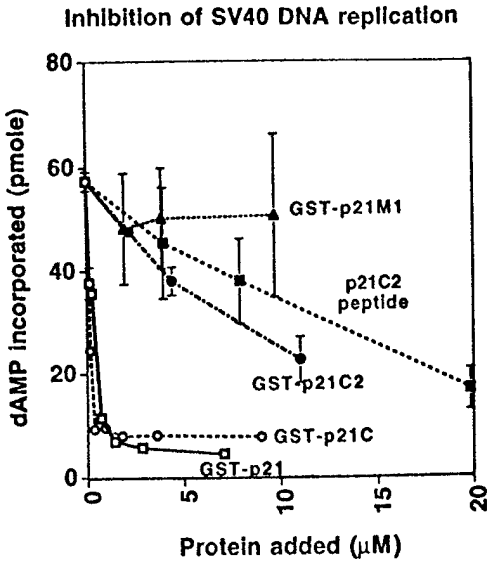

Figure 3

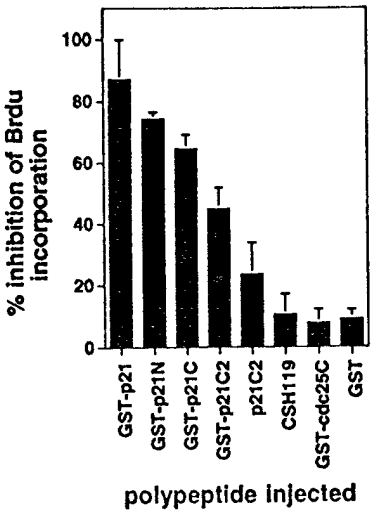

Figure 4

CONCLUSIONS

L22Q-W23S p53 showed decreased transcription activation, loss of transcription repression, wild-type RPA binding and decreased growth suppression, indicating that transcriptional trans-activation and/or transcription repression is most important for growth suppression. RPA binding, in contrast, lost in the D48H-D49H and W53S-F54S alleles of p53, appears unimportant for growth suppression.

p53 has other functions relevant to the production of cancers. It is required to induce apoptosis in response to x-irradiation or chemotherapy, to produce a pause in DNA replication after a sub-lethal dose of radiation so as to give the cell time to repair its DNA, and to prevent gene amplification. p53 can induce apoptosis through a pathway independent of new mRNA transcription and protein synthesis , making it likely that the transcriptional trans-activation function of p53 is occasionally dispensable for this activity. p53 has recently been shown to selectively bind to insertion-deletion mismatch lesions, and by analogy with the XP-A-RPA interaction, may be involved in recruiting RPA to these sites of DNA repair. The p53-RPA interaction could be important for apoptosis induction and/or the other functions of p53 described in this paragraph.

In conclusion we have determined which feature of p53 and other trans-activators promotes interaction with RPA, and shown with two mutant alleles of p53 that growth suppression occurs independent of binding to RPA. We have also shown that trans-activation by p53 is affected by mutations at residues 22-23, and this allele is most defective in growth suppression. These results suggest that while RPA-p53 interaction is not important, transcription trans-activation by p53 is important for cell growth suppression.

Although p53 binds Rpa1 without displacing Rpa2, it excludes single-stranded DNA from the complex. The domain mapping results suggest that the regions of Rpa1 necessary for binding p53 include the N terminal 221 amino acids and possibly additional residues in the 411-492 region. Therefore p53 could potentially exclude DNA from the N terminal low affinity DNA binding site (residues 1-219 of Rpa1). Exclusion of DNA from the high affinity DNA binding site (278-492 of Rpa1) could be due to a selective overlap of the DNA and p53 binding sites in the 411-492 region or due to conformation changes induced in Rpa1 by p53. The absence of significant overlap between the p53 and Rpa2 binding sites of Rpa1 explains why p53 does not exclude Rpa2-3 from the p53-Rpa1 complex. Recombinant peptides derived from simple direct repeats of 10-12 amino acid sequences containing bulky hydrophobic residues interspersed with negatively charged residues bind RPA well . Such sequences are commonly noted in "acidic activation domains" of transcription trans-activators like p53 and VP16. Mutations in bulky hydrophobic residues of p53 abolish interaction with RPA . Therefore, the domain of RPA which binds to p53 is likely responsible for more generalized interactions of RPA with other proteins containing "acidic activation domains" like VP16, yeast Gal4, and the DNA repair protein XP-G.

278-616 RPA was not bound by p53 and simultaneously lost the ability to support DNA replication. This result indicates that the domain of Rpa1 which interacts with p53 (and other acidic activators) could be crucial for the replication promoting functions of RPA. In such a case, it may be difficult to generate a mutant version of RPA which does not bind p53 and yet supports DNA replication. Of course, finer mutations in the 1-277 region of Rpa1 may allow us to separate these two aspects of RPA function. However, we are reconsidering whether this is a worthwhile goal in the context of breast cancer, mostly because the RPA-p53 interaction does not appear to be important for growth suppression by p53 (as shown above).

In contrast, the correlation of growth suppression by p53 with transcription activation is clear. Consequently we plan to focus our attention on one of the effectors of p53, p21, which is transcriptionally induced by p53. We have defined a 39 amino acid fragment of p21 which is sufficient to bind the DNA replication factor PCNA with high

affinity (Kd = 10-20 nM) (20). This peptide can inhibit DNA replication in vitro, and microinjection of a GST fusion protein containing this domain inhibited S phase in vivo. The DNA replication enzymes are attractive targets for development of new agents for chemotherapy. We examined the p21-PCNA interaction with the long term goal of determining if it can be exploited for design of drugs which reach their target (PCNA) in vivo. As a first approximation we used a peptide (p21C2) derived from p21 which interacted with PCNA and inhibited SV40 replication reaction in vitro. A ten fold higher concentration of GST-p21C2 or the free p21C2 peptide was required to inhibit the SV40 replication reaction compared to GST-p21C. This is likely to be due to the 100 fold decrease in affinity of p21C2 for PCNA at the physiological temperature, although we cannot rule out the existence of factors in cell extracts that specifically interfere with the action of p21C2 but not p21C.

The efficacy of p21 based peptides at reaching and inhibiting PCNA in vivo was not clear before the present study. Because GST-p21C2 effectively inhibited cell growth, but the free p21C2 peptide did not, we suspect that smaller peptides are unlikely to be useful for inhibiting PCNA in vivo. However the high affinity of the interaction between GST-p21C and PCNA (Kd of 10-20 nM) suggests that this interaction is suitable for pharmacological purposes. For comparison other protein-protein interactions which have the potential for development as therapeutic agents include the inhibition of cyclin-cdk kinases by p21 (Ki = 1 nM) the interaction between phosphotyrosine containing peptides and SH2 domains (Kd = 10-100 nM), and the interaction between SH3 domains and proline rich peptides (Kd = 1000 nM).

In general peptide based therapeutics suffers from the obvious problem of delivering peptides into cells at high concentrations. Our results point to two additional drawbacks : decreasing the length of interacting peptide rendered the interaction thermodynamically unstable, and additional poorly understood mechanisms were responsible for the small p21C2 peptide, but not GST-p21C2 protein, being inactivated in the cell. A small chemical that can mimic the structure of the active PCNA binding region of p21C2 may overcome all these drawbacks. Such a chemical may also be used to target other replication inhibitors to the site of DNA synthesis. Therefore the best approach will be to determine the structure of the p21C2 binding interface and design chemicals which mimic the same.

We have made significant progress in the project. The studies on the RPA-p53 association address the key question we set out to answer: is the interaction necessary for growth suppression by p53? The answer was no, which forced us to re-consider which function of p53 was most important for growth suppression. The ability to activate transcription was the answer. Since p21 was recently identified as a key target for the transcription activation by p53, we looked closely into how p21 inhibits cell growth. Inhibition of cyclin-cdk kinases was very important and we have followed up on the mechanism for this inhibition. Although these results are not discussed in my report, they are discussed in Dr. J. Chen's report. In my report I have followed up on whether p21 impinges on the DNA replication apparatus in vitro and in vivo, through the inhibition of PCNA (as a new task added to this year's report). Here, the answer was yes, and we have explored whether this interaction can be exploited by trying to make a small peptide based on the structure of p21 which can inhibit PCNA in vitro. In vivo, however, the peptide was not as effective as a longer 90 amino acid sequence, probably because of thermo-lability of the interaction of PCNA with the smaller peptide. These results indicate that the most direct way to exploit the p21-PCNA interaction for therapeutics will be by getting the crystal structure of the complex and then attempting to synthesize small chemicals which mimic the effect of p21 on PCNA.

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Dissection of Functional Domains of the Human DNA Replication Protein Complex Replication Protein A*

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Replication protein A (RPA) is a mammalian single-stranded DNA binding factor essential for DNA replication, repair, and recombination. It is composed of three subunits of 70, 34, and 13 kDa (Rpa1, Rpa2, and Rpa3, respectively). Deletion mapping of the Rpa2 subunit identified the domain required for interaction with Rpa1 and Rpa3 which does not include the N-terminal domain that is phosphorylated during S phase. Deletion mapping of Rpa1 defined three domains. The C-terminal third of the Rpa1 polypeptide binds Rpa2 which itself forms a bridge between Rpa1 and Rpa3. The N-terminal third of Rpa1 bound single-stranded DNA under low stringency conditions only (0.1 M NaCl), while a central domain binds to single-stranded DNA under both low and high stringency conditions (0.5 M NaCl). Binding to p53 requires the N-terminal third of Rpa1 with some contribution from the C-terminal third. The evolutionarily conserved putative zinc finger near the C terminus of Rpa1 was not required for binding to single-stranded DNA, Rpa2, or p53. However, all three subdomains of Rpa1 and the zinc finger were essential for supporting DNA replication *in vitro*. These experiments are a first step toward defining peptide components responsible for the many functions of the RPA protein complex.

RPA[1] is absolutely required in the *in vitro* SV40-based DNA replication reaction (1–3) and is also important for many other DNA-mediated processes. It is required for replication in *Xenopus* egg extracts, for the successful passage of yeast through the large budded stage corresponding to S phase, for excision repair of pyrimidine dimers, and for recombination (4–7). The three-subunit RPA complex binds to single-stranded DNA and modulates the function of DNA polymerases α and δ. It also physically interacts with the SV40 origin-binding protein T antigen and with DNA polymerase α (8–12). In addition RPA associates with proteins containing acidic transcriptional activator domains such as p53, VP16, and the DNA repair protein XP-G. These interactions have been proposed to inhibit DNA binding by RPA and to recruit RPA for replication and repair (13–16). Interaction with p53 is of particular interest because RPA bound to p53 failed to bind single-stranded DNA (15).

The 70-kDa subunit (Rpa1) can bind to single-stranded DNA on its own but cannot support DNA replication. The 34-kDa subunit (Rpa2) is phosphorylated in a cell cycle-dependent manner by multiple kinases which include the cdk kinases and the DNA-dependent protein kinase (17–19). The phosphorylation is also induced by x-irradiation of cells, and this form of phosphorylated RPA fails to support DNA replication (20). We have recently discovered a homolog of the middle subunit, Rpa4, which is expressed selectively in some quiescent tissues apparently uncomplexed with Rpa1 and Rpa3 (21). In order to understand how the various subunits of RPA interact with each other, how RPA binds DNA, and how the activity of the protein may be regulated by protein-protein interactions and post-translational modifications, we have mapped the functional domains of the three subunits of RPA using cloned cDNAs coding for the human RPA subunits (22–24). Gomes and Wold (25) have used C-terminal deletions of Rpa1 to map regions required for binding to single-stranded DNA and to Rpa2/Rpa3. This report confirms and extends their results.

Three approaches have been taken for studying the mutant forms of RPA subunits. The first uses the yeast two-hybrid/interaction trap method for studying protein-protein interactions between the subunits. The second uses proteins made by *in vitro* transcription and translation in co-immunoprecipitation and binding assays to analyze protein-protein or protein-nucleic acid interactions. The third uses recombinant RPA holocomplexes to confirm the findings from the earlier assays and to determine the domains of Rpa1 essential for SV40-based DNA replication. Together, these results provide a functional map of the RPA complex and suggest that, beside the binding of single-stranded DNA and the recruitment of Rpa2 and 3 to the replication apparatus, the Rpa1 subunit executes additional functions essential for DNA replication.

MATERIALS AND METHODS

Plasmid Constructions—The plasmids important for this study are listed in Table I. pEGRPA1 has been described (15). A DNA fragment containing the coding region of RPA3 was made by PCR from phRPA3 and cloned between *Eco*RI and *Xho*I sites of pEG202 to make pEGRPA3. pJGRPA3 was made by transferring the *Eco*RI-*Xho*I fragment of pEGRPA3 to pJG 4–5. Yep-RPA2 has been described as pJM403 (5). PCR was performed with appropriate primers to synthesize a DNA fragment that contained the coding region of RPA2 (from phRPA2) flanked by *Bam*HI sites. This PCR product was cloned into the *Bam*HI site of pKS$^+$ such that the RPA2 reading frame was oriented in the same direction as the *lacZ* gene to make p102. After making p102, the entire RPA2 reading frame was sequenced with multiple primers to ensure that there were no PCR-induced mutations.

The *Bam*HI fragment of p102 was cloned into pEG202 to obtain pEGRPA2. The deletions in RPA2 were made as follows. pEGRPA2 was cut with *Nco*I (sites in RPA2 and in the polylinker of EG202 downstream from RPA2) and ligated, to obtain pEG107. The *Nco*I fragment

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[1] The abbreviations used are: RPA, replication protein A; SV40, Simian virus 40; GST, glutathione *S*-transferase; PCR, polymerase chain reaction; mAb, monoclonal antibody.

TABLE I
Plasmids

The amino acid numbering is from the published sequence of Rpa1 and Rpa2. Thus full-length Rpa1 contains residues 1–616 and full-length Rpa2 contains residues 1–270. Deletions result in shorter fragments, *e.g.* amino acids 1–492 of Rpa1, or a missing central fragment, *e.g.* Δ222–411 Rpa1 is missing amino acids 222–411. Point mutations result in amino acid changes, thus C500S indicates that the cysteine at position 500 is changed to serine and so forth. "Base plasmid" indicates the plasmid which was primarily modified (by deletion, insertion or mutation) to create the indicated plasmid.

Plasmid	Relevant genotype	Rpa1/Rpa2/Rpa3 fragment	Base plasmid
pEG202	*His*3; lexA under control of an ADH promoter		
pSH18-34	*URA*3; *lacZ* gene under control of 8 lexA operators		
pJG4-5	*TRP1*; transcriptional activation domain fused to inserted protein under control of a GAL1 promoter		
pYEP51	*LEU*2; inserted gene expressed under control of a GAL10 promoter		
pEGRPA1	LexA fused to Rpa1	1–616, Rpa1	pEG202
pEGRPA2	LexA fused to Rpa2	1–270, Rpa2	pEG202
pEGRPA3	LexA fused to Rpa3	1–121, Rpa3	pEG202
pEGΔ222–411	LexA fused to Rpa1	1–222+412–616, Rpa1	pEG202
pEG457–616	LexA fused to Rpa1	457–616, Rpa1	pEG202
pEG1–492	LexA fused to Rpa1	1–492, Rpa1	pEG202
pYEP51-RPA2	Rpa2	1–270, Rpa2	YEP51
pJGRPA2	Activation domain fused to Rpa2	1–270, Rpa2	pJG4–5
pJG121	Activation domain fused to Rpa2	43–270, Rpa2	pJG4–5 Activation
pJG109	Activation domain fused to Rpa2	102–270, Rpa2	pJG4–5 Activation
pJG115	Activation domain fused to Rpa2	134–270, Rpa2	pJG4–5 Activation
pJG118	Activation domain fused to Rpa2	163–270, Rpa2	pJG4–5 Activation
pJG117	Activation domain fused to Rpa2	1–163, Rpa2	pJG4–5 Activation
pJG116	Activation domain fused to Rpa2	1–134, Rpa2	pJG4–5 Activation
pJG107	Activation domain fused to Rpa2	1–101, Rpa2	pJG4–5 Activation
pJG124	Activation domain fused to Rpa2	43–163, Rpa2	pJG4–5 Activation
phRPA2	Rpa2 under control of a T7 promoter	1–270, Rpa2	pKS
phRPA3	Rpa3 under control of a T7 promoter	1–120, Rpa3	pKS^-
phRPA1	Rpa1 under control of a T7 promoter	1–616, Rpa1	pKS^-
prevRPA1	Rpa1 under control of a T3 promoter	1–616, Rpa1	pKS^+
phRPA1ΔCla	Rpa1 under control of a T7 promoter	1–616, Rpa1Δ from ClaI site in 3′-untranslated region	phRPA1
p1–221	Rpa1 under control of a T7 promoter	1–221, Rpa1	phRPA1
p1–309	Rpa1 under control of a T7 promoter	1–309, Rpa1	phRPA1
p1–492	Rpa1 under control of a T7 promoter	1–492, Rpa1	phRPA1
pΔ222–411	Rpa1 under control of a T7 promoter	1–221+412–616, Rpa1	phRPA1ΔCla
p278–616	Rpa1 under control of a T7 promoter	278–616, Rpa1	phRPA1ΔCla
p1–372	Rpa1 under control of a T3 promoter	1–372, Rpa1	prevRPA1
p1–522	Rpa1 under control of a T3 promoter	1–522, Rpa1	prevRPA1
p349–616	Rpa1 under control of a T3 promoter	349–616, Rpa1	prevRPA1
pm1–616	Rpa1 under control of a T7 promoter	1–616, Rpa1 (with C500S, C503S)	phRPA1
p11dtRPA	Rpa1, 2, and 3 under control of a T7 promoter	1–616, Rpa1 1–270, Rpa2 1–121, Rpa3	pET11d
pm11dtRPA	Rpa1, 2, and 3 under control of a T7 promoter	1–616, Rpa1 (with C500S, C503S) 1–270, Rpa2 1–121, Rpa3	pET11d
p11dtRPA (Δ222–411)	Rpa1, 2, and 3 under control of a T7 promoter	1–221+412–616, Rpa1 1–270, Rpa2 1–121, Rpa3	pET11d
p3atRPA (278–616)	Rpa1, 2, and 3 under control of a T7 promoter	278–616, Rpa1 1–270, Rpa2 1–121, Rpa3	pET3a

containing the C-terminal part of RPA2 from the same digestion was cloned into pEG202 (B8) (21) to obtain pEG109. p102 was linearized by partial digestion with *Bam*HI, and then cut in the RPA2 coding region with *Bgl*II. After ligation the plasmid containing the C-terminal part of RPA2 from the *Bgl*II site was obtained (p113). The *Eco*RI-*Bam*HI fragment from p113 was cloned into pEG202 to obtain pEG115. p102 was linearized with *Bgl*II, the ends filled in with Klenow polymerase, and blunt ends ligated to obtain p102ΔBg which has a four base pair insertion that disrupts the RPA2 reading frame at the *Bgl*II site. The *Bam*HI fragment from p102ΔBg was cloned into the *Bam*HI site of pEG202 to obtain pEG116. The N-terminal 560-base, and the C-terminal 330-base, *Bam*HI to *Bcl*I fragments of p102 were cloned into the *Bam*HI site of pEG202 to obtain pEG117 and pEG118 respectively. PCR with appropriate primers was used to make a DNA fragment encoding RPA2 from A43 to the C terminus this fragment was digested with *Eco*RI and *Bam*HI or *Eco*RI and *Bcl*I. The 755-base *Eco*RI-*Bam*HI fragment and the 420-base *Eco*RI-*Bcl*I fragment was cloned between *Eco*RI and *Bam*HI sites of pEG202 to obtain pEG121 and pEG124, respectively. To make the pJG derivatives which express the deletions of RPA2 fused to the acidic activation domain, *Eco*RI-*Xho*I fragments from each of the pEG plasmids described above were cloned between the *Eco*RI and *Xho*I sites of pJG4–5 (except for pJG121, for which the *Eco*RI-*Sal*I fragment from pEG121 was transferred to pJG4–5). As an additional safeguard against unintentional mutations in the RPA2 coding region, pJGRPA2 was also made by a second strategy. A DNA fragment containing the RPA2 coding region was synthesized by PCR and cloned between the *Bam*HI and *Eco*RI sites of EG202, and the *Eco*RI-*Xho*I fragment from this secondary plasmid transferred to JG4–5 to make the second version of pJGRPA2.

The deletion derivatives of Rpa1 were made as follows. p1–616 was the original phRPA1 clone obtained from Dr. T. Kelly where the RPA1 cDNA is cloned into pKS^- between *Eco*RI sites such that the RPA1 gene is downstream from the T7 promoter. The *Eco*RI fragment was recloned into pKS^+ in the reverse orientation to obtain prevRPA1. phRPA1 was cut with *Cla*I (sites in the untranslated region downstream from RPA1 and in the polylinker) and ligated to obtain phRPA1ΔCla. phRPA1 was cut with *Hin*dIII or with *Xho*I and ligated to obtain p1–219 and p1–309, respectively. phRPA1ΔCla was cut with *Hin*dIII and ligated to obtain pΔ222–411. phRPA1 was cut with *Bcl*I and *Eco*RV (in the polylinker), the ends filled in and ligated to obtain p1–492. prevRPA1 was cut with *Xba*I or with *Ssp*I and ligated to obtain p1–372 and p1–522, respectively. prevRPA1 was cut with *Xho*I and ligated to obtain p349–616,

and phRPA1ΔCla was cut with *Pst*I and ligated to obtain p278–616.

The pEG202-based constructs expressing deletions of RPA1 were made as follows. pEG1–616 is the same as pEGRPA1. *Nco*I-*Xho*I fragment of pΔ222–411 and *Nco*I-*Sal*I fragment of p1–492 were cloned between *Nco*I and *Xho*I sites of pEG202(B8) to obtain pEGΔ222–411 and pEG1–492, respectively. A DNA fragment of RPA1 coding for residues 457–616 was synthesized by PCR with the appropriate primers and cloned between *Eco*RI and *Bam*HI sites of pEG202 to obtain pEG457–616.

The plasmids for expressing RPA holocomplex with deletions or mutations in Rpa1 were derived from p11dtRPA which expressed wild-type human RPA in bacteria and which were provided by Dr. Marc Wold (26). pm11dtRPA was cloned by exchanging the *Nco*I-*Ssp*I fragment of the RPA1 coding region of p11dtRPA with that of pm1–616 (see below, "Site-directed Mutagenesis of RPA1"). The internal *Hin*dIII fragment of RPA1 coding region of p11dtRPA was deleted to obtain p11dtRPAΔ222–411. RPA1 amino acid 278–403 flanked by *Nde*I and *Bam*HI sites was generated by PCR with appropriate primers and cloned in pET3a. The *Xho*I-*Bam*HI fragment of this plasmid was replaced by the *Xho*I-*Bam*HI fragment of p11dtRPA to obtain p3aRPA1 278–616. RPA3-containing *Eco*RI fragment from p11dtRPA and RPA2-containing *Bam*HI fragment from p11dtRPA were successively cloned into p3aRPA1 278–616 to obtain p3atRPA278–616. The region of Rpa1 generated by PCR was sequenced to rule out any secondary mutations.

Mutant proteins are named to indicate the amino acids that are present in the derivative. The exceptions are Δ222–411 Rpa1, which indicates the missing internal amino acids, and m1–616 Rpa1, which indicates a version of Rpa1 with point mutations (described below).

Site-directed Mutagenesis of RPA1—The Stratagene PCR site-directed mutagenesis kit was used to generate a plasmid expressing m1–616 Rpa1 (pm1–616). Primer RPA70 S3V4 (5′-AAATTCGGTGTC-GACCTTCTCAGAGCGGTA CAATCC-3′), is complementary to human RPA1 sequence 1555–1590 with underlined nucleotides changed from the wild-type sequence. Thus two of the four evolutionarily conserved cysteines in Rpa1 (amino acids 500 and 503) are both changed to serine. Primer 1591–1621 is the same as the corresponding sequence of human RPA1. Primer 1591–1621 was 5′-phosphorylated, and 15 pmol of each primer was used for PCR on 0.5 pmol of phRPA1 template DNA using *Taq* polymerase and *Taq* extender. The PCR cycling parameters are as follows: segment 1, 1 cycle of 94 °C 4 min, 50 °C 2 min, 72 °C 2 min; segment 2, 8 cycles of 94 °C 1 min, 56 °C 2 min, 72 °C 1 min; segment 3, 1 cycle of 72 °C 5 min. By keeping the number of cycles low the chances of unintentional PCR-induced mutation are decreased. The PCR reaction is thus used to create a linear DNA fragment corresponding to the whole phRPA1 plasmid except the mutations incorporated in the primers. 1 μl of *Dpn*I (which cuts methylated template DNA) and *Pfu* DNA polymerase were added at 37 °C for 30 min to simultaneously select against the parental template DNA and to polish the ends of products, respectively. After heat inactivation of the enzymes (72 °C for 30 min) the PCR product was circularized by ligation and transformed into *Escherichia coli*. The resulting plasmids were screened for the incorporation of the *Sal*I site designed in the mutagenic primer, and candidate plasmids were sequenced to confirm the mutation and rule out adjoining secondary mutations.

Yeast Transformations and β-Galactosidase Assays—*Saccharomyces cerevisiae* strain EGY40 (*MAT***a** *trp1 ura3 his3 leu2*) or EGY48 (*MAT***a** *trp1 ura3 his3 LEU2::pLexAop6-LEU2*) (27) were transformed with the various plasmids, and transformed yeast was selected by the appropriate nutritional markers according to standard protocols. Briefly, the JG plasmids contain *TRP1*, the EG plasmids contain *HIS3*, and the *lacZ*-containing plasmids pSH18–34 contains the *URA3* marker. β-Galactosidase assays were done on log phase cultures by standard protocols and presented in units using the formula A_{420}/(minutes to develop the $A \times$ yeast protein in μg). All assays presented in a given figure were done at the same time so that the results are comparable within a figure.

In Vitro Transcription-Translation and Binding Assays—Radiolabeled protein was synthesized with [^{35}S]methionine and the Promega TnT coupled *in vitro* transcription-translation kit. For single-stranded DNA binding, 20 μl of translation mix was brought up to 200 μl of buffer A7.4 (20 mM Tris HCl, pH 7.4, 1 mM EDTA, 10% glycerol, 0.01% Nonidet P-40, 25 mM NaCl) supplemented with NaCl to the required concentration (500 mM or 100 mM). The lysate was incubated with 20 μl of single-stranded DNA cellulose (1:3 slurry in the same buffer) for 1 h at 4 °C, and washed five times with 1 ml of the same buffer. Bound proteins were eluted by boiling the DNA-cellulose pellet in Laemmli's buffer, electrophoresed on 15% SDS-polyacrylamide gel and visualized by fluorography in 1 M sodium salicylate.

For immunoprecipitation, 10 μl of the translation products were precleared by incubating for 1 h with 20 μl of protein A-Sepharose beads in 200 μl of buffer A7.4. The precleared lysate was recovered from the beads by centrifugation and immunoprecipitated by incubation with the appropriate primary and secondary antibodies followed by protein A-Sepharose beads. The beads were washed with four times in 1 ml of NETN buffer (0.5% Nonidet P-40, 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA), and immunoprecipitated proteins were analyzed as described above for single-stranded DNA-bound proteins.

For measuring the binding of Rpa1 to p53, the translation mixes were diluted 1:10 with NETN containing 50 mM NaCl and 10 mg/ml Blotto. After incubation for 1 h at 4 °C with glutathione agarose beads coated with approximately 1 μg of glutathione *S*-transferase (GST) or GST-p53, the beads were washed with four times with 1 ml of NETN (50 mM NaCl). Bound proteins were visualized as described above.

When recombinant RPA complexes with mutant forms of Rpa1 were tested for ability to bind single-stranded DNA or p53, the assays were the same as above, except that 100 ng of RPA were used as input, and the bound Rpa1 and Rpa2 were visualized by immunoblotting.

Glycerol gradient analyses of the translation mixes were done in buffer A7.4 or in radioimmune precipitation buffer (10 mM, Tris, pH 7.4, 5 mM EDTA, 150 mm NaCl, 10% glycerol, 0.1% SDS, 0.5% deoxycholate, 1% Triton X-100, 1% Trasylol) as described in Keshav *et al.* (21).

Immunoblotting—Yeast strains expressing various EG or JG derivatives were lysed, and the protein lysates were immunoblotted with anti-lexA (for EG proteins), 12CA5 monoclonal antibody to the hemagglutinin epitope present on all JG fusion proteins, and/or monoclonal antibodies to human Rpa1 (p70–9) or Rpa2 (p34–20) to ensure that proteins of the correct size were made.

Expression and Purification of Recombinant RPA—The proteins were expressed and purified as described previously (26). The protein complexes were named according to the Rpa1 mutant present in the complex.

Nitrocellulose DNA Binding Assay—The recombinant RPA complex proteins were incubated with ^{32}P-labeled poly(dA) for 5 min at 37 °C in a 25-μl reaction mix (replication conditions including replication buffer). The reaction mix was then passed through alkali-treated nitrocellulose paper (0.5 M KOH 20 min, H_2O, 5 min, 0.1 M Tris, pH 7.5, 40 min) by suction in a Dot-blot apparatus, followed by washing with three times with 200 μl of wash buffer (25 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 50 mM NaCl). The nitrocellulose paper was then air-dried and cut into squares for scintillation counting. Each point was done in duplicate and the results presented as percentage of input DNA.

SV40 Based DNA Replication Reaction—Replication of a plasmid containing an SV40 origin of DNA replication was carried out in human cell extracts depleted of RPA (1), which have been supplemented with the bacterially expressed RPA holocomplexes (26).

RESULTS

Protein-Protein Interactions between the RPA Subunits—The yeast two-hybrid system (27) was used to analyze interactions among the RPA subunits. In this system a DNA binding protein (lexA) is fused to the protein of interest (Rpa1 in EgRpa1 or Rpa3 in EgRpa3). This protein cannot activate a specially designed promoter upstream from a *lacZ* gene unless a second protein is present which contains a generic transcriptional activation domain fused to a domain that interacts stably with the first fusion protein.

The *S. cerevisiae* strain EGY40 carrying the reporter plasmid pSH18–34 (*lacZ* gene under control of a lexA operator) and pEGRPA1 (expresses a fusion between the lexA DNA binding domain and human Rpa1 protein) did not have significant β-galactosidase activity. Plasmid pJGRPA2 expresses a protein containing a synthetic transcriptional activation domain fused to human Rpa2. When pJGRPA2 was transformed into the yeast (EGY40::pSH18–34, pEGRPA1), and the expression of the JgRpa2 fusion protein induced with galactose, significant β-galactosidase activity was produced (Fig. 1). This suggests that the JgRpa2 fusion protein interacted with the EgRpa1 fusion protein at the lexA operator and created a transcription activator at the *lacZ* promoter. In similar experiments, JgRpa2 also interacted with the EgRpa3 fusion protein, but JgRpa3 did not interact with the EgRpa1 fusion protein. These results support a model where the Rpa2 subunit interacts with both

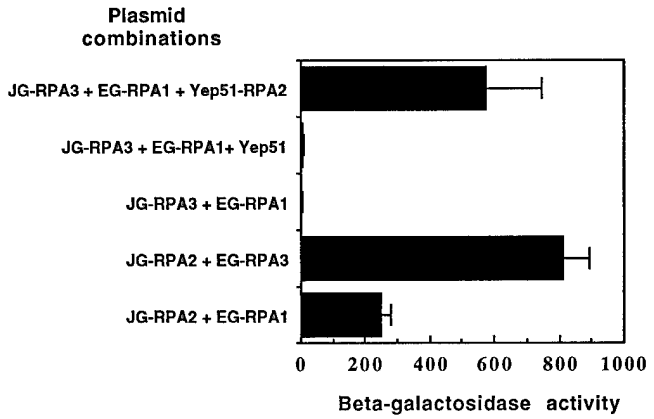

FIG. 1. **β-Galactosidase activity of lysates of yeast containing the indicated plasmids expressed in units described in the text.** All the strains contain pSH18–34 and are grown on galactose containing medium to induce the expression of the genes carried on JG and Yep plasmids. The values are averages from at least two independent colonies and the error bar indicates the standard deviation. Significant enzyme activity indicates interaction (direct or indirect) between the proteins expressed from the JG and EG plasmids.

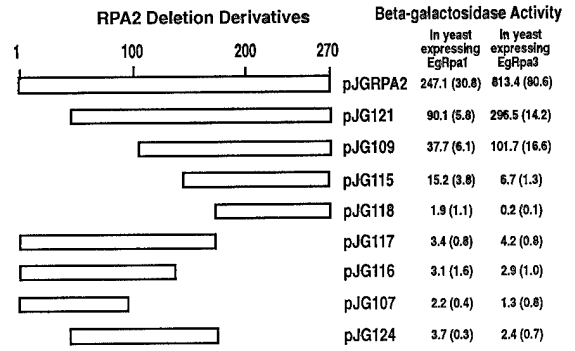

FIG. 2. **β-Galactosidase activity of lysates of yeast containing either pEGRPA1 (interaction with RPA1) or pEGRPA3 (interaction with RPA3) and the indicated JG plasmids expressing various derivatives of RPA2.** Averages of at least two different colonies are presented (in units described in the text) with the standard deviations in parentheses. Significant β-galactosidase activity indicates association between the proteins expressed from the pJG and pEG plasmids.

the Rpa1 and the Rpa3 subunit and thus has the potential to form a bridge between the other subunits.

This model was tested directly by adding the Rpa2 protein in trans to the two hybrids, EgRpa1 and JgRpa3. If Rpa2 forms a bridge between Rpa1 and Rpa3, one would predict that while EgRpa1 and JgRpa3 fail to interact with each other on their own, the expression of Rpa2 in these strains of yeast would result in a EgRpa1-Rpa2-JgRpa3 interaction establishing a functional transcription activator at the *lacZ* promoter. Yeast carrying the pSH18–34 plasmid was transformed with pJGRPA3, pEGRPA1 and either a control vector Yep51, or Yep51-RPA2, which expresses the human Rpa2 protein under the control of a GAL10 promoter. Comparison of beta-galactosidase activities of strains harboring Yep51 *versus* Yep51-RPA2 showed that Rpa2 does form a bridge between EgRpa1 and JgRpa3 (Fig. 1).

Region of Rpa2 Involved in Interaction with Rpa1 and Rpa3—Several deletion derivatives of Rpa2 were cloned into JG4–5 vector so that JgΔRpa2 fusion proteins were expressed in yeast containing pSH18–34 and either pEGRPA1 or pEGRPA3. β-Galactosidase activities produced in the various strains of yeast are presented in Fig. 2. A derivative of Rpa2 with a deletion of the first 42 amino acids lost 60% of β-galactosidase activity obtained with full-length Rpa2 (when either was co-expressed as a Jg fusion protein with either EgRpa1 or EgRpa3). The significant residual interaction suggests that the N-terminal 42 amino acids of Rpa2, which includes the sites of phosphorylation, are outside the minimal domain involved in interaction with Rpa1 or Rpa3. Further deletion into Rpa2 sequence showed that the N-terminal limit of the minimal domain that interacts with Rpa1 or Rpa3 is between amino acids 102 and 134. On the C-terminal side, deletion of the last 108 amino acids abolished interaction with both Rpa1 and Rpa3. Thus the C-terminal boundary of the Rpa1/Rpa3 interacting domain of Rpa2 is likely to be C-terminal to amino acid 162, although without finer mutations we cannot rule out the possibility that the large deletion denatured the Rpa2 protein. The deletions of Rpa2 affected interaction with both Rpa1 and Rpa3 to the same degree so that the Rpa1 and Rpa3 binding regions of Rpa2 were not separated in this deletion analysis.

Region of Human Rpa1 Necessary for Interacting with Rpa2—A selected series of deletions in human RPA1 gene were cloned into pEG202 vector to obtain the corresponding EgΔRpa1 derivatives. When the interaction of each of these derivatives with JgRpa2 was tested (Fig. 3), we found that a C-terminal deletion that truncates the putative zinc finger of Rpa1 (1–492) completely abolishes the interaction, while a deletion of amino acids 222–411 of Rpa1 still retained considerable interaction. Thus the region of Rpa1 from amino acid 412 to the C terminus is necessary for interacting with Rpa2 in this assay.

When human Rpa1 was synthesized in a rabbit reticulocyte lysate, a monoclonal antibody to human Rpa2, p34–20, specifically immunoprecipitated the 70 kDa Rpa1 subunit (Fig. 4*A*, *lane 3*). This was not due to cross-reaction of the p34–20 antibody with p70, because (i) the antibody did not recognize p70 on an immunoblot (data not shown), and (ii) boiling of the translation mixes to disrupt Rpa1-Rpa2 interactions prevented co-immunoprecipitation of Rpa1 by the p34–20 antibody (data not shown).

RPA is routinely found in the cytoplasmic fractions when cells are disrupted. Therefore rabbit Rpa2 could be present in the reticulocyte lysate and p34–20 could co-immunoprecipitate Rpa1 by virtue of its association with the rabbit protein. Glycerol gradient sedimentation showed that when co-translated *in vitro*, human Rpa1, 2, and 3 co-sedimented as a broad peak of 5–8 S consistent with a complex appropriate to the size of RPA holocomplex (120–150 kDa). When Rpa2 and 3 were translated in the absence of Rpa1 they co-sedimented at 3–4.6 S, consistent with a Rpa2/Rpa3 complex of 45–70 kDa. When translated on its own in rabbit reticulocyte lysates Rpa1 still sediments as a broad peak of 5–8 S consistent with the size of a Rpa1-2-3 complex. The Rpa1 from all fractions could be precipitated by anti-Rpa2 antibody. Taken together, these results suggest that even when Rpa1 is translated on its own, it forms a complex with unlabeled rabbit Rpa2 + 3 from the reticulocyte lysates.

One would then predict that the domain of Rpa1 shown in the two-hybrid interaction assay to be important for association with Rpa2 will also be important for co-immunoprecipitation of *in vitro* translated Rpa1 by p34–20 antibody. Several deletion derivatives of Rpa1 were synthesized in the rabbit reticulocyte lysate, and immunoprecipitation by p34–20 examined (Fig. 4*B* and summarized in Fig. 8). As expected from the results of the yeast interaction trap, co-precipitation by p34–20 antibody was seen with the Δ222–411 derivative of Rpa1 (*lane 7*) but not with the 1–492 derivative (*lane 10*). The 1–522 derivative was weakly associated with rabbit Rpa2, although the significance of this association is unclear. The absence of any association of the 1–372 derivative, and of strong association of the 349–616 protein, again emphasizes that the C-terminal part of Rpa1 is

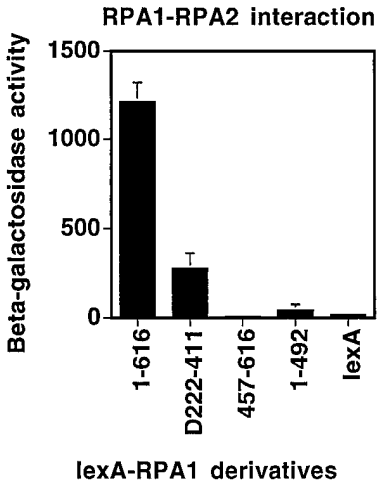

FIG. 3. **β-Galactosidase activity of lysates of yeast containing pJGRPA2 and the indicated EG based plasmids expressing lexA or lexA fusion derivatives.** *1–616*, lexA fused to full-length Rpa1; *D222–411*, *lexA* fused to Δ222–411 Rpa1; *457–616*, lexA fused to amino acids 457 to the C terminus of Rpa1; *1–492*, lexA fused to Rpa1 from the N terminus to amino acid 492.

important for association with Rpa2. The minimal domain is confirmed to be between amino acids 412 and 616.

Region of Rpa1 Involved in Binding Single-stranded DNA—RPA can bind to single-stranded DNA at salt concentrations in excess of 0.5 M NaCl, and we were interested in determining which portions of Rpa1 were necessary for this high avidity binding of DNA. Full-length Rpa1 (1–616) was synthesized *in vitro* in rabbit reticulocyte lysates (containing rabbit Rpa2 and 3), bound to single stranded DNA cellulose matrix and washed with 0.5 M NaCl. The binding of Rpa1 to single-stranded DNA was resistant to elution by 0.5 M NaCl (Fig. 5*A*, *lane 2*, and summarized in Fig. 8). Both 1–522 and 1–492, which bound Rpa2 very poorly or not at all, could still bind single-stranded DNA at 0.5 M NaCl (Fig. 5*B*, *lanes 6* and *10*). 1–372 was poor in single-stranded DNA binding activity (Fig. 5*B*, *lane 4*) putting the C-terminal limit of the DNA binding region between 372 and 492. 278–616 could, while 349–616 could not, bind single-stranded DNA (Fig. 5, *A*, *lane 8*, and *B*, *lane 2*), putting the N-terminal end of the DNA binding domain between residues 278 and 349. 349–616 or Δ222–411 derivatives of Rpa1 could not bind single-stranded DNA at 0.5 M NaCl (Fig. 5*B*, *lanes 2* and *8*), although they were both capable of associating with Rpa2. We conclude that for binding single-stranded DNA at 0.5 M NaCl, Rpa1 requires a minimal domain between amino acids 278 and 492.

If the NaCl concentration of the DNA binding reaction and of the washes is reduced to 0.1 M NaCl, the N-terminal part of Rpa1 (1–309 or 1–219) now binds to single-stranded DNA, although this association is sensitive to 0.5 M NaCl (Fig. 5*C*). Therefore, Rpa1 has a second single-stranded DNA binding domain (amino acids 1–219), but binding of DNA through this domain is disrupted by high salt concentrations.

The absence of background "binding" of Rpa1 to the negative control CL6B beads argues against the nonspecific aggregation of the *in vitro* translated proteins on single-stranded DNA cellulose beads. To rule out the possibility that the binding to DNA was secondary to the association of Rpa1 with an unknown DNA binding protein from rabbit reticulocyte lysates, selected recombinant RPA complexes containing deletion derivatives of Rpa1 were purified to homogeneity and tested in a nitrocellulose filter binding assay (Fig. 7*B*). The results corroborate those obtained in Fig. 5.

Region of Rpa1 Required for Binding p53—We have reported

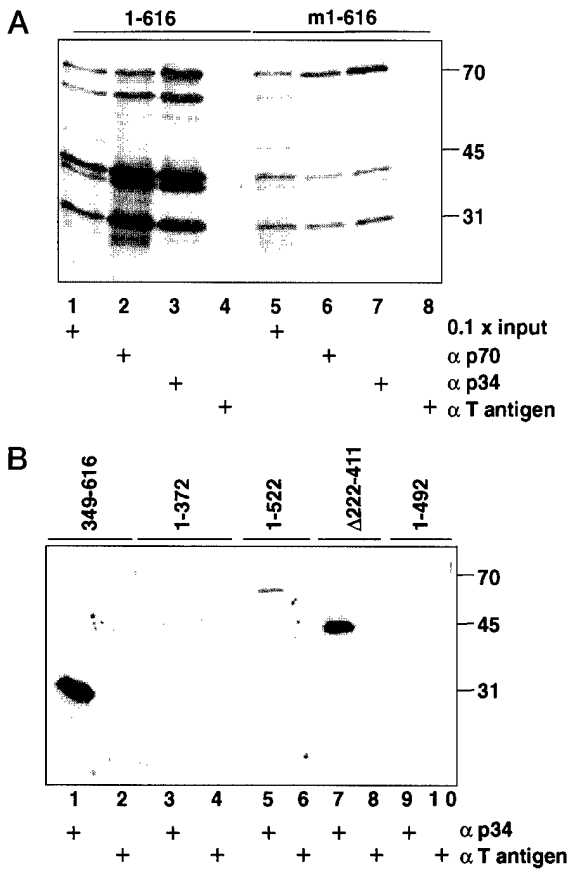

FIG. 4. **Immunoprecipitation of radiolabeled human Rpa1 and its derivatives after synthesis in rabbit reticulocyte lysates was followed by fluorography to visualize the precipitated radiolabeled proteins.** The precipitation of Rpa1 with anti-Rpa2 antibody indicates that the Rpa1 derivative associates with rabbit Rpa2. *A*, the antibodies used were mAb p34–20 (*αp34*) or mAb 419 (*αT antigen*, negative control) or mAb p70–9 (*αp70*, positive control). One-tenth of the proteins input into the reactions is shown in the lanes marked *0.1x input*. The largest size protein in the input lanes corresponds to full-length translation product, while the smaller proteins are derived from aberrant initiation from internal methionines. *1–616*, wild-type Rpa1; *m1–616*, Rpa1 with a mutation in the zinc finger. *B*, the same experiment performed with additional derivatives of Rpa1. Only the immunoprecipitates with mAb p34–20 (test) and mAb 419 (negative control) are shown. The input proteins can be seen in the input lanes in Fig. 5*B*.

that RPA bound to p53 fails to bind single-stranded DNA (15). One explanation could be that the overlapping regions of Rpa1 are required to bind the two ligands, so that the ligands are mutually exclusive. To determine if this was the case, we used the deletion derivatives of Rpa1 to map the region required to bind to p53. Rpa1 and deletion derivatives were synthesized *in vitro* and bound to glutathione agarose beads coated with either GST or GST-p53 (Fig. 6*A*, summarized in Fig. 8). A small C-terminal deletion increased binding to p53 (1–616 *versus* 1–522, *lanes 3* and *21*). This result was obtained consistently and could indicate that the C-terminal 94 amino acids of Rpa1 somehow interfere with association with p53. Comparison of 1–492 to 1–372 (*lanes 6* and *15*) suggests that the C-terminal limit of the p53 binding region lies between 372 and 492. 1–278 is important for the binding of p53 because 278–616 is unable to bind p53. The requirement for this region is emphasized by the comparison of the p53 binding activity of 1–522 Rpa1 *versus* 278–522 Rpa1 (*lanes 12* and *21*). However, the 1–278 region alone did not bind p53 (*e.g.* 1–309, *lane 18*).

We confirmed and extended the results using purified RPA holocomplexes with selected deletion derivatives of Rpa1 (Fig. 7*A*, described below) and analyzed for binding to p53 (Fig. 6*B*).

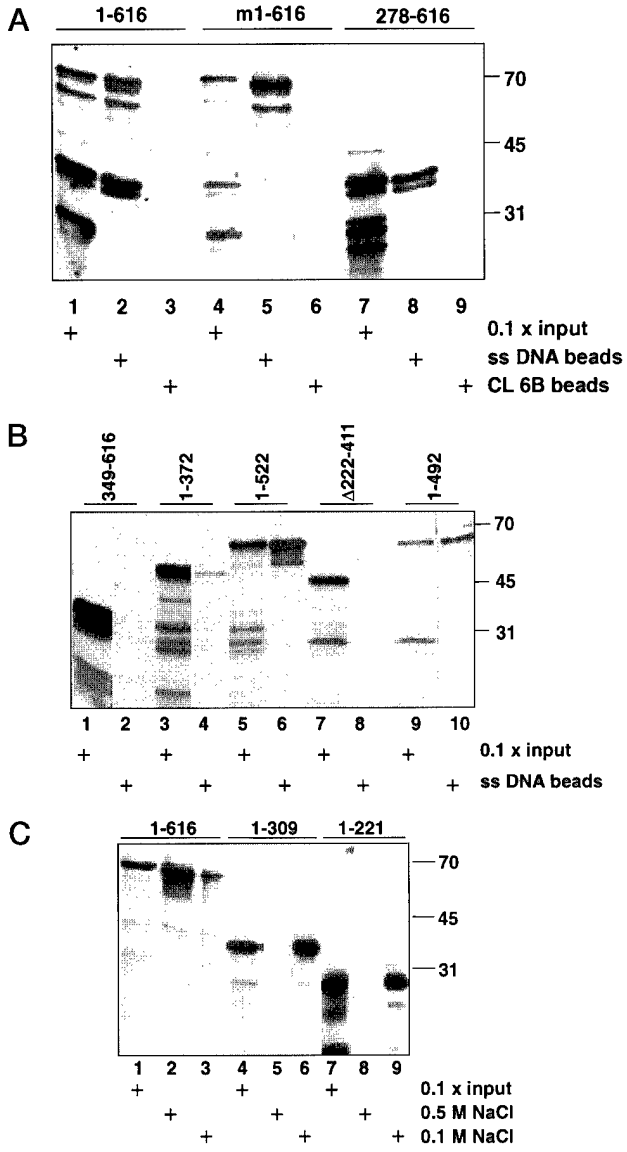

FIG. 5. **Radiolabeled Rpa1 and its derivatives were synthesized by *in vitro* transcription-translation in rabbit reticulocyte lysates, and visualized by fluorography as in Fig. 5, except that the ability of the Rpa1 derivatives to bind single-stranded DNA is being tested here.** *A*, The proteins bound to single-stranded DNA cellulose beads at 0.5 M NaCl are shown alongside one-tenth of the proteins input into the binding reactions and the proteins binding to negative control Sepharose CL6B beads. Full-length Rpa1 (*1–616*) and mutant derivatives used in the reactions are indicated above the lanes. *B*, additional mutant derivatives of Rpa1 were tested for binding single-stranded DNA cellulose beads. One-tenth of the proteins input into each of the reactions is shown in parallel lanes. None of the proteins bound to the negative control Sepharose CL-6B beads (data not shown). *C*, at lower salt concentration (0.1 M NaCl), the N-terminal portion of Rpa1 (amino acids 1–221 and 1–309) bound to single-stranded DNA cellulose. The proteins bound to single-stranded DNA cellulose at 0.5 M or 0.1 M NaCl are shown next to one-tenth of the input proteins. None of the proteins bound to the negative control Sepharose CL-6B at either salt concentration (not shown).

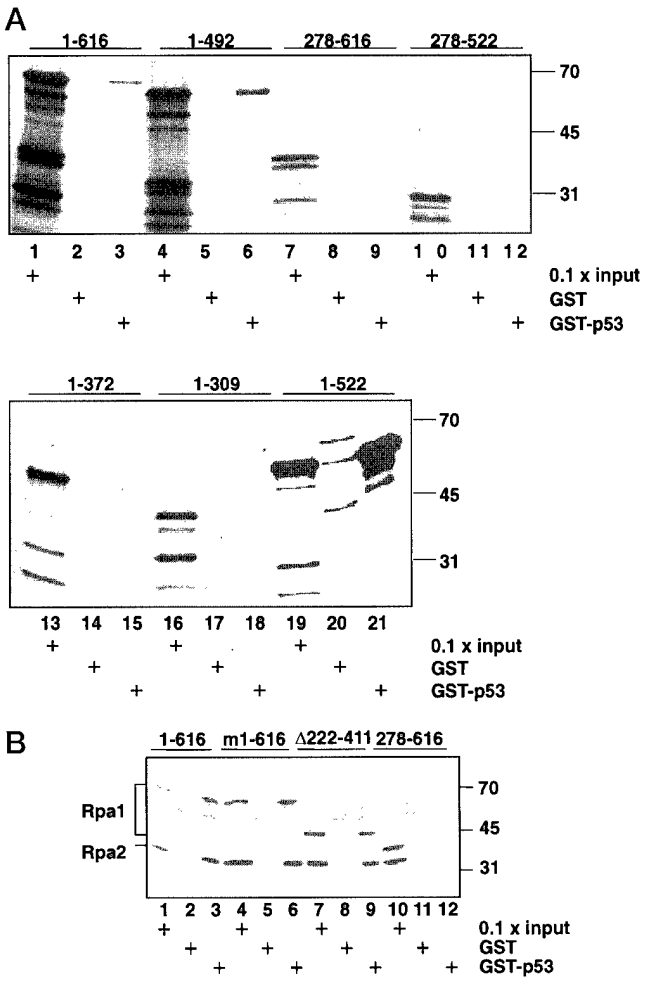

FIG. 6. **Binding of Rpa1 and derivatives to GST-p53 and to GST is shown alongside the input proteins.** *A*, radiolabeled Rpa1 synthesized by *in vitro* transcription-translation in rabbit reticulocyte lysates was incubated with glutathione Sepharose beads coated with indicated proteins and bound Rpa1 visualized by fluorography. One-tenth of input protein, protein bound to GST-p53 coated beads and protein bound to GST coated (negative control) beads are shown in parallel lanes. The Rpa1 derivatives used are indicated above the lanes. *B*, selected deletion or point-mutated derivatives of Rpa1 were synthesized in bacteria along with Rpa2 and Rpa3, the RPA holocomplexes purified (see Fig. 7*A*), and their ability to bind GST-p53 specifically tested. In this case the Rpa1 and Rpa2 proteins present in the indicated lanes were visualized by immunoblotting with mAb p70–9 (αRpa1) and mAb p34–20 (αRpa2), respectively.

RPA with Δ222–411 Rpa1 bound to p53 indicating that the middle third of Rpa1 was not required for this activity (Fig. 6*B*, *lane 9*). The failure of RPA with 278–616 Rpa1 to bind p53 (Fig. 6*B*, *lane 12*) is consistent with the results in Fig. 6*A* (*lane 9*) and confirms that the N-terminal 1–278 amino acids of Rpa1 are essential for the interaction. Taken together, we conclude that the N-terminal 221 amino acids of Rpa1 together with residues in the 411–492 region are sufficient for binding p53.

We previously reported that the N-terminal 1–308 amino acids of Rpa1 is sufficient for p53 binding (15) in contradiction to the data shown in Fig. 6*A*. The discrepancy between the two results may be due to differences in the way Rpa1 was produced. In the previous study, lexA-Rpa1(1–308) fusion protein expressed in yeast was used for binding to GST-p53, while in the current experiments we used Rpa1 produced in rabbit reticulocyte lysates without any N-terminal fusion for its association with GST-p53. In the previous study the lexA portion of lexA-Rpa1(1–308) may stabilize a weak interaction by artificially increasing the protein-protein contact area between the two proteins, but such a concern does not arise in the current study. Alternatively, in the current study the rabbit reticulocyte lysate may contain proteins which competitively inhibit a weak interaction of 1–308 Rpa1 with GST-p53. Indeed, Rpa1Δ222–411 produced in rabbit reticulocyte lysates was poor at interacting with GST-p53 (data not shown), although bacterially produced recombinant RPA Δ222–411 containing the same derivative of Rpa1 associated strongly with GST-p53 (Fig. 6*B*). Therefore, while we can conclude that the N-terminal

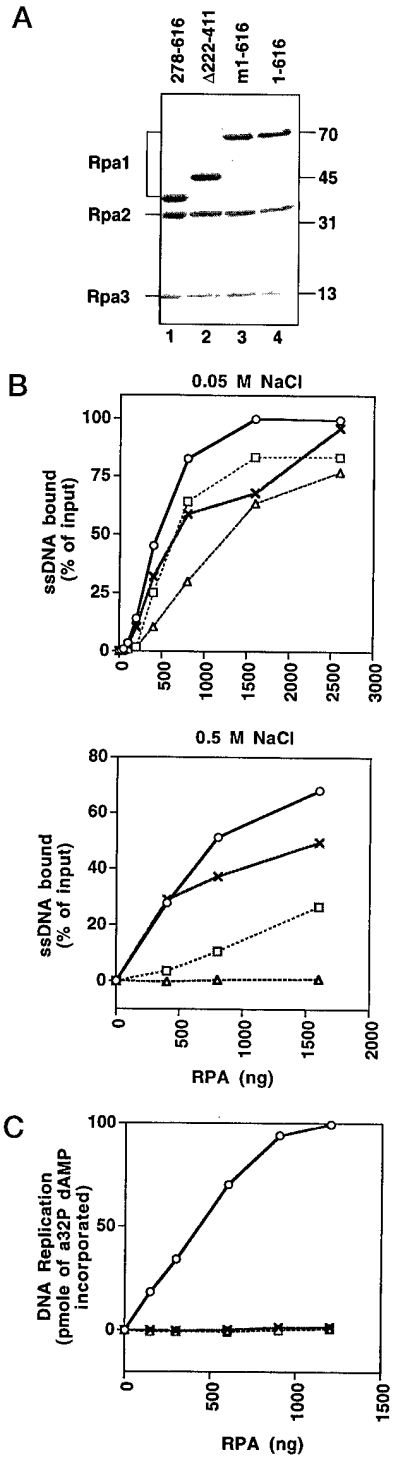

FIG. 7. **RPA holocomplexes prepared with selected mutants of Rpa1 were tested for their DNA binding and DNA replication activities.** *A*, Coomassie stain of 2 μg of each RPA holocomplex containing Rpa2, Rpa3, and indicated versions of Rpa1. *B*, binding of recombinant RPA to single-stranded DNA in a nitrocellulose filter binding assay. *Top*, a nitrocellulose filter based DNA binding assay was done in replication buffer in a 25-μl reaction, and washes were done with a buffer containing 50 mM NaCl. The *y* axis indicates the percentage of input poly(dA) bound by the indicated amounts of RPA. *Bottom*, binding and washes were done at 0.5 M NaCl. *C*, DNA replication was studied in an SV40-based *in vitro* reaction using T antigen, 293 S100 cell extract selectively depleted of endogenous RPA, and indicated amounts of recombinant RPA complexes in a 25-μl reaction. Picomoles of [α-^{32}P]dAMP incorporated into polynucleotide (in a theoretical 50-μl reaction) measure the extent of DNA replication. For *B* and *C*, the RPA holocomplexes are: 1–616 RPA (*circles*), m1–616 RPA (*crosses*), 278–616 RPA (*squares*), and Δ222–411 RPA (*triangles*).

278 amino acids of Rpa1 are required for interacting with GST-p53, we cannot yet conclude whether it is sufficient or insufficient for the interaction.

The Putative Zinc Finger of Rpa1 Is Dispensable for Binding Single-stranded DNA, p53, or Rpa2—A putative C4-type zinc finger motif was noted at position 481–503 of human Rpa1, which is evolutionarily conserved in yeast Rpa1 (5, 6, 23). The ability of 1–492 (which deletes 2 of the 4 cysteines) to bind single-stranded DNA suggests that the zinc finger is not required for binding to single-stranded DNA. Since 1–492 could bind p53, the zinc finger is also dispensable for binding p53. On the other hand, binding to Rpa2 was fairly weak with 1–522, and nonexistent when the C-terminal deletion reaches the zinc finger (1–492). To determine whether the zinc finger was important for binding Rpa2, point mutations were made in the Rpa1 cDNA which changed the 2 C-terminal cysteines of the putative zinc finger to serines (m1–616). This point-mutated form of Rpa1 was synthesized *in vitro*, and its ability to bind Rpa2 was measured by co-immunoprecipitation with monoclonal antibody p34–20 (Fig. 4*A*, *lane 7*). The mutated form of Rpa1 associated with Rpa2 as effectively as wild-type Rpa1. Also, as expected from the deletion derivatives, binding to single-stranded DNA and p53 was unaffected by the loss of the putative zinc finger (Fig. 5*A*, *lane 5*; Fig. 6*B*, *lane 6*). Therefore, the putative zinc finger is not required for binding any of the three ligands tested, Rpa2, single-stranded DNA, and p53.

Regions of Rpa1 Required for Supporting DNA Replication—Several studies have demonstrated that even though Rpa1 alone binds to single-stranded DNA, it fails to support SV40-based DNA replication, suggesting the importance of Rpa2 and Rpa3 for DNA replication. Therefore any mutation in Rpa1 which affects its ability to form the holocomplex renders it inactive in DNA replication, making the C-terminal third of Rpa1 essential for the process. The domain mapping experiments now allowed us to selectively create mutant forms of Rpa1 which still form a holocomplex with Rpa2 and Rpa3, and then test the ability of the mutants to support DNA replication. We purified RPA holocomplexes from bacteria containing (*a*) 1–616 Rpa1 (wild-type Rpa1), (*b*) m1–616 Rpa1 (with a mutation in the zinc finger), (*c*) 278–616 Rpa1 (which has lost the ability to interact with acidic activation domains of trans-activator proteins like p53), and (*d*) Δ222–411 Rpa1 (which has lost the high affinity DNA binding site). All four forms of Rpa1 associated with Rpa2 and Rpa3. Fig. 7*A* shows the pure RPA holocomplexes. DNA binding by the mutant RPA holocomplexes in the DNA cellulose pull-down assays was as predicted from the domain mapping experiments (data not shown). RPA with wild-type, zinc finger- mutated, and 278–616 Rpa1 were all able to bind to DNA in high and low salt concentrations. RPA with Δ222–411 Rpa1 could bind to single-stranded DNA in low but not in high salt concentration.

DNA binding by these holocomplexes was quantitated by a nitrocellulose DNA binding assay (Fig. 7*B*; binding was in replication conditions and washes in a buffer of 0.05 M NaCl in the top). m1–616 RPA and 278–616 RPA retained considerable DNA binding ability (60–70% of wild-type RPA activity respectively at 48 ng/μl), while Δ222–411 RPA was weaker in its DNA binding capacity (40% of wild-type activity at 48 ng/μl). At high salt concentration of binding and washing (0.5 M NaCl), both wild-type and mutant RPA complexes bound less DNA compared to low salt conditions (Fig. 7*B*, *bottom*). m1–616 RPA and 278–616 RPA had 70 and 40% of the activity of wild-type RPA (respectively; 48 ng/μl), while Δ222–411 RPA did not bind any DNA, consistent with the results obtained in Fig. 5*B*.

The RPA holocomplexes were tested for their ability to support SV40-based DNA replication in an extract depleted of

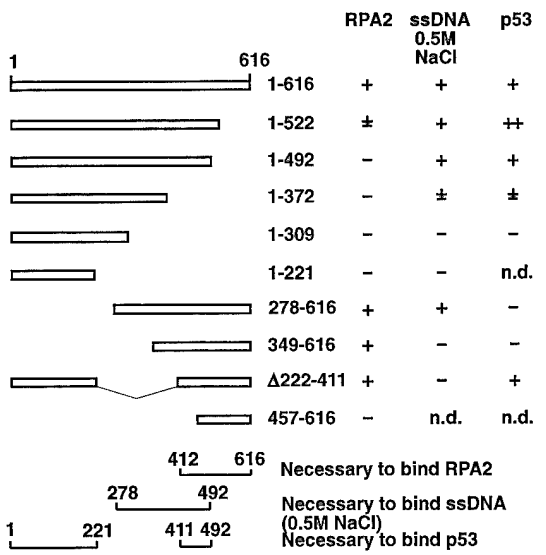

FIG. 8. **Summary of binding data of Rpa1 to Rpa2, p53, and single-stranded DNA cellulose (the last only at 0.5 M NaCl).** +, similar to wild-type Rpa1; −, no binding; ±, weak binding; ++, better than wild-type Rpa1; *n.d.*, not determined. The minimal regions of Rpa1 required for the three activities are indicated at the bottom. The salt-sensitive DNA binding site which binds single-stranded DNA only at less than 0.1 M NaCl is located at amino acids 1–219.

endogenous RPA. Despite the fact that the three mutant RPA holocomplexes bound significant amounts of single-stranded DNA in the salt and RPA concentrations used in the replication reaction, none of them supported DNA replication (Fig. 7*C*). Both m1–616 RPA and 278–616 RPA bound single-stranded DNA to an extent comparable with that of wild-type RPA but did not support any DNA replication. This result suggests that DNA replication requires additional activities from Rpa1 beside binding to single-stranded DNA and formation of the RPA holocomplex.

DISCUSSION

In order to understand how the trimeric RPA protein complex participates in DNA replication, we have determined some of the functional regions of the Rpa1 and Rpa2 proteins. The C-terminal part of Rpa1 binds to Rpa2 and the middle part binds to single-stranded DNA in a salt resistant manner (Fig. 8). Surprisingly, the N-terminal part of Rpa1 also binds to single-stranded DNA, but only at low salt concentrations. p53 uses amino acids from both the N- and C-terminal portion of Rpa1 to bind to the protein. The C-terminal two-thirds of Rpa2 forms a bridge between Rpa1 and Rpa3, although additional contacts between Rpa1 and 3 cannot be ruled out.

Attempts to reconstitute the RPA holocomplex by expression of recombinant polypeptides have suggested that although Rpa1 and Rpa2 can interact to form an easily dissociable complex, Rpa2 and Rpa3 form a stable complex which will associate with Rpa1 to form the Rpa1–2-3 complex (26, 28). We have modified the two-hybrid system to demonstrate that Rpa2 is capable of forming a bridge between Rpa1 and Rpa3. This report is the first demonstration that bridging interaction in a three subunit complex can be studied by a modification of the two-hybrid system. Deletion mapping of Rpa2, however, did not separate the parts of the protein that interact with Rpa1 or Rpa3. Finer mutagenesis may be required to achieve this goal, and since we now have a genetic system for assaying the interaction, it should be possible to mutagenize randomly the cDNA coding for the Rpa2 protein and screen for mutations that selectively affect interaction with Rpa1 or Rpa3. The N-terminal 43 amino acids of Rpa2 containing its sites of phosphorylation by cdk kinases (17) appears to be independent from the region required to associate with Rpa1 and 3.

Rpa1 was readily divisible into regions important for one function but not another. The deletions could potentially disrupt the tertiary structure of Rpa1 or produce insoluble proteins. However, since various deletion derivatives of Rpa1 carried out some functions and not others, we believe that the deletions do not result in a global denaturation of the protein. Also the bead-binding assays were confirmed by two-hybrid assays in yeast and by solution binding assays with soluble recombinant proteins to eliminate artifacts due to insoluble deletion derivatives. The regions indicated in Fig. 8 are required for binding the indicated ligands. The Rpa1-Rpa2 interaction requires the C-terminal one-third of Rpa1, a result consistent with that obtained by Gomes and Wold (25). This region also appears sufficient for association with Rpa2. Although this domain assignment utilized the association of human Rpa1 with rabbit Rpa2 in the reticulocyte lysate the results are the same for the association of human Rpa1 and Rpa2 when the deletions were tested (*a*) in the yeast two-hybrid assay and (*b*) for the ability to form the RPA holocomplex when co-expressed with Rpa2 and 3 in bacteria.

Approximately the middle third of Rpa1 (278–492) is important for binding of single-stranded DNA in 0.5 M NaCl, a conclusion reached from the properties of 278–616 (+), Δ222–411 (−), 1–492 (+) and 1–372 (±). Definition of the minimal regions of Rpa1 required to bind Rpa2 and single-stranded DNA is also supported by the binding properties of the smaller products obtained during *in vitro* transcription-translation of Rpa1 (Fig. 4*A*, *lane 1*). Assuming that these products are by initiation from internal methionines, polypeptides of 57, 36, and 29 kDa probably correspond to products initiating at methionines 97, 278, and 349. Since Rpa2 is expected to bind to the C-terminal region of these products, immunoprecipitation with anti-Rpa2 antibody should precipitate all the smaller products, consistent with what is observed (Fig. 4*A*, *lane 3*). Single-stranded DNA cellulose however, only binds to products containing 278–492, and therefore should bind only to the internally initiated products of 57 and 36 kDa but not of 29 kDa. This is in fact what is observed (Fig. 5*A*, *lane 2*). The DNA binding region of Rpa1 does not contain the zinc finger motif or residues 109–145, the reported region of sequence similarity to *E. coli* single-stranded DNA binding protein (ssb) (23).

DNA binding by Rpa1 at 0.15 M NaCl in a Southwestern assay showed that small N-terminal fragments of Rpa1 (*e.g.* 1–249, 1–326, and so forth) were capable of binding DNA (25). It is likely that the DNA binding these authors observe in the Southwestern assay is the equivalent of the DNA binding we see at 0.1 M NaCl, and can be executed by a salt-sensitive DNA binding site near the N terminus of Rpa1. Gomes and Wold (25) reported a significant drop in affinity when the deletions removed amino acids N-terminal to residue 441 (1–441 had high and 1–326 had low association constants for DNA). The close agreement on the C-terminal limit of the DNA binding region when assaying binding in high salt *versus* binding with high association constant (residue 492 *versus* 441) suggests that the salt resistant DNA binding is due to the high affinity binding site. Since 278–616 Rpa1 binds to DNA at high salt, we suggest that the salt-resistant (high affinity?) DNA binding site in the middle of Rpa1 is separate from a salt-sensitive DNA binding site contained in the 1–219 region of the protein.

The existence of separate salt-sensitive and -resistant DNA binding sites on Rpa1 was unsuspected. RPA has been reported to bind to single-stranded DNA in two different modes: one with each RPA molecule covering 8 bases, and the other with each RPA molecule covering 30 bases (29, 30). Different parts of

the Rpa1 molecule with different DNA binding sites could be involved in the two modes of DNA binding. The deletion mapping also shows that the Rpa2 binding region of Rpa1 is dispensable for binding single-stranded DNA with high affinity, implying that Rpa2–3 are not necessary to give Rpa1 a specific structure essential for high affinity association with DNA.

In vitro DNA replication reactions are performed at low salt concentrations (less than 50 mM KCl), conditions under which the low affinity DNA binding site is functional. However, the results reported in Fig. 7 suggest that the salt-sensitive DNA binding site (absent in 278–616, but present in Δ222–411 Rpa1) is required but not sufficient to support DNA replication. Of course, the salt-sensitive DNA binding site in the N-terminal third of Rpa1 could actually be used in replication to bind a negatively charged protein and not DNA.

Scrutiny of the Rpa1 deletions shows that association with p53 did not correlate with binding to single-stranded DNA or to Rpa2. There was a derivative which bound p53 well but not single-stranded DNA (Δ222–411), others which bound both well (1–522), and a third which bound DNA well but not p53 (278–616). Likewise there were Rpa1 derivatives which bound p53 well but not Rpa2 (1–522 and 1–492), and a derivative which did the reverse (278–616). This confirms our previous observation that the Rpa1-p53 interaction required neither Rpa2 nor single-stranded DNA (15). The importance of the N-terminal 278 amino acids of Rpa1 for p53 binding is confirmed by a darker exposure of the autoradiogram in Fig. 6*A*: internally initiated polypeptides in the 1–616, 1–522, and 1–492 input lanes corresponding in size to those expected from initiation at methionine 278 were not bound by GST-p53.

Although p53 binds Rpa1 without displacing Rpa2, it excludes single-stranded DNA from the complex (15). The domain mapping results suggest that the regions of Rpa1 necessary for binding p53 include the N-terminal 221 amino acids and possibly additional residues in the 411–492 region. Therefore p53 could potentially exclude DNA from the N-terminal low affinity DNA binding site (residues 1–219 of Rpa1). Exclusion of DNA from the high affinity DNA binding site (278–492 of Rpa1) could be due to a selective overlap of the DNA and p53 binding sites in the 411–492 region or due to conformation changes induced in Rpa1 by p53. The absence of significant overlap between the p53 and Rpa2 binding sites of Rpa1 explains why p53 does not exclude Rpa2–3 from the p53-Rpa1 complex. Recombinant peptides derived from simple direct repeats of 10–12 amino acid sequences containing bulky hydrophobic residues interspersed with negatively charged residues bind RPA well.[2] Such sequences are commonly noted in "acidic activation domains" of transcription trans-activators like p53 and VP16. Mutations in bulky hydrophobic residues of p53 abolish interaction with RPA.[2] Therefore, the domain of RPA which binds to p53 is likely responsible for more generalized interactions of RPA with other proteins containing "acidic activation domains" such as VP16, yeast Gal4, and the DNA repair protein XP-G.

The ability to divide the Rpa1 subunit into subdomains required for essential activities (holocomplex formation and DNA binding) opened the way toward determining whether other subdomains of Rpa1 are essential for DNA replication. Δ222–411 Rpa1 did not support DNA replication indicating that the evolutionary conserved salt-resistant DNA binding activity present in all single-stranded DNA binding replication proteins is essential for replication. The 278–616 and m1–616 form of Rpa1, however, carried out the core activities of the protein: binding to DNA in high salt concentrations and forming a holocomplex with Rpa2 and Rpa3. Yet, neither of these derivatives supported DNA replication suggesting that other activities of Rpa1 are essential for replication. At present we cannot determine whether these mutations selectively remove a functional domain of Rpa1 required only for DNA replication, or selectively misfold the RPA complex such that DNA binding is allowed but not DNA replication. In either case, determination of the step in the DNA replication reaction blocked with these mutants will shed light on what additional activities or properties are required from RPA to support DNA replication.

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[2] L. M. Leiter and A. Dutta, unpublished observations.

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Loss of transactivation and transrepression function, and not RPA binding, alters growth suppression by p53

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The tumor suppressor protein p53 activates transcription from promoters with specific p53 binding elements, represses transcription from promoters without such elements and interacts with and inhibits the single-stranded DNA binding activity of the human DNA replication factor RPA. All these activities involve the N terminal 70 amino acids of p53. Dissection of the domains of p53 which bind RPA suggest that multiple sub-domains of the protein synergize to give strong RPA binding. Point-mutations in one of these sub-domains of p53 significantly diminish its ability to interact with RPA. A multimer of a peptide from p53 which includes these residues, or of a peptide from the acidic activation domain of the prototypic trans-activator protein VP16, can itself bind to RPA. Comparison of sequences of these multimeric peptides suggests that aromatic amino acids flanked by negatively charged residues are important for binding RPA. Several alleles of p53 with point mutations in the N terminal region were analysed for their relative abilities to bind RPA, activate or repress transcription, and suppress growth of p53 null SaOs2 and H1299 cells. Both mutants of p53 with decreased RPA binding suppressed cell growth as well as wild-type p53, suggesting that p53 can suppress growth without interacting with RPA. The allele that lost most of the transcription activation function also lost most of its transcription repression activity suggesting that interaction with the same basal transcription factors are involved in both functions. This same allele bound RPA well but was defective in growth suppression. Therefore, transcription activation and/or repression appear to be more important for the growth suppression function of p53 than RPA binding.

Keywords: p53; RPA; transcription; cell cycle; DNA replication

Introduction

Since its discovery in 1979, many investigators have convincingly demonstrated that p53 has a critical role in the cell. By halting abnormal cell division, p53 can suppress the uncontrolled growth that leads to neoplasia. Overexpression of the wild type p53 protein arrests cell growth just before the onset of DNA replication at the G1-S boundary. Wild type p53 is essential for G1 arrest following radiation-induced DNA damage, or for apoptosis of the cell if the DNA damage is extensive (Kastan *et al.*, 1991; Kuerbitz *et al.*, 1992). Also, wild type p53 suppresses the potential of a cell to amplify portions of its genome (Livingstone *et al.*, 1992; Yin *et al.*, 1992). The transforming mutants of p53 are defective in all these functions. Therefore a major concern in the field of cancer research is how the wild type p53 protein carries out these diverse functions: inhibition of S phase, pause in DNA replication following DNA damage, induction of apoptosis and inhibition of DNA amplification.

Three mechanisms have been proposed by which p53 inhibits DNA replication. First, p53 could act as a suppressor of S phase by the sequence specific transcriptional induction of genes (Fields *et al.*, 1990; Raycroft *et al.*, 1990; Pietenpol *et al.*, 1994) that negatively regulate cell growth e.g. the p21 gene (El *et al.*, 1993; Gu *et al.*, 1993; Harper *et al.*, 1993; Xiong *et al.*, 1993; Noda *et al.*, 1994). Indeed, several authors to date have found a correlation between transcription activity and p53's ability to suppress growth (Crook *et al.*, 1994; Pietenpol *et al.*, 1994). Second, p53 can more generally repress transcription from certain cellular promoters (Seto *et al.*, 1992; Mack *et al.*, 1993; Crook *et al.*, 1994; Subler *et al.*, 1994). Since these promotors do not contain p53 binding sites, it is thought that p53 may reduce transcription by binding to and sequestering basal transcription factors. The role of p53's transrepression activity in growth suppression is less consistent in the literature, however. Third, p53 interacts with the SV40 T antigen, inhibits the helicase activity of T antigen and inhibits the SV40 based *in vitro* DNA replication (Friedman *et al.*, 1990). Therefore p53 could potentially interact with and inhibit a cellular origin binding replication initiator protein or replication helicase (as yet unidentified). This is supported by the recent report by Cox *et al.* (1995) that p53 inhibited nuclear DNA replication in a transcription-free DNA replication extract from Xenopus eggs.

We and others reported that p53 interacts with a cellular DNA replication factor RPA (Dutta *et al.*, 1993; Li *et al.*, 1993). RPA (RF-A or human ssb) is a complex of three polypeptides of 70, 34 and 13 kD, essential for SV40 DNA replication *in vitro* (Wobbe *et al.*, 1987; Fairman *et al.*, 1988; Ishimi *et al.*, 1988; Wold *et al.*, 1988; Tsurimoto *et al.*, 1989) and also excision repair in animal cells (Coverley *et al.*, 1991). The 70 kD subunit from human cells binds to single-stranded DNA, and supports unwinding of the SV40 origin. RPA from *S. cerevisiae* is also composed of polypeptides of similar molecular weight, and the genes for each of the subunits are essential for viability (Brill *et al.*, 1989, 1991; Heyer *et al.*, 1990), indicating that

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RPA will also be essential for human chromosomal replication. In our previous experiment, the interaction with p53 prevented RPA from binding to ssDNA and, therefore, suggested a fourth mechanism by which p53 could inhibit DNA replication.

In the present study, we have mapped the RPA binding site of p53 and found that mutations in amino acids 53 and 54 (from tryptophan and phenylalanine to serine residues) in the N terminus abolish RPA binding. RPA was previously shown to bind to the transcriptional activation domains of Herpes Simplex virus transactivator VP16, of the yeast trans-activator Gal4 and the Bovine Papilloma virus trans-activator E2 (He *et al.*, 1993; Li *et al.*, 1993). Interestingly, we demonstrate a similar binding motif in the VP16 RPA binding domain. We have compared the ability of the 53,54 mutant to suppress growth in SaOs2 and H1299 cells with a mutant deficient in both transactivation and transrepression functions. The mutant unable to bind to RPA but which retained both transactivation and transrepression functions retained growth suppression activity comparable to that of wild type p53. However, a mutant defective in both transactivation and transrepression (amino acids 22,23) functions significantly lost growth suppressive activity compared to that of wild type. The loss of p53's transcription activity with mutations in 22,23 residues is known (Lin *et al.*, 1994). However, to our knowledge, this is the first report that this same mutant also significantly loses transrepression activity from a CMV promoter, a fact that should be considered when attributing biological functions of p53 solely to the loss of transactivation function by this mutant.

These studies suggest that RPA binding by p53 may be less critical in growth suppression than its ability to activate or repress transcription in SaOs2 and H1299 cells. However, the involvement of the interaction in other cellular processes, e.g. DNA repair, apoptosis, have not been studied and deserve further investigation, especially since p53-induced transcription is dispensible for many of these functions.

Results

Sub-domains of p53 synergize to give strong RPA binding

GST fusion proteins containing various fragments of p53 were generated, bound to glutathione agarose beads and their ability to bind RPA examined by affinity chromatography (Figure 1). Fragments of p53 are named by the position of the amino acids in the complete p53 sequence. We have shown earlier that two domains of p53, N2 (amino acids 2–121) and 5C (amino acids 289–393), could independently bind RPA. The domain containing amino acids 2–71 of p53 had equivalent RPA binding activity as 2–121 (data not shown). However, further dissection of sub-domains containing amino acids 2–45 or 46–71 showed much reduced RPA binding activity. Ten times as much of each GST-sub-domain protein (e.g. GST 2–45 or GST 46–71) were compared to GST-domain protein (GST 2–71) in their ability to bind RPA. The binding activity of each sub-domain was less than one-tenth that of the corresponding domains (Figure 1b). Thus the better binding of RPA by a domain (e.g. 2–71) is probably not a simple summation of RPA binding by each of the sub-domains (e.g. 2–45 and 46–71). Similarly, at the C terminal end, the domain containing amino acids 289–356 had significant RPA binding; but sub-domains 289–330 or 331–356 did not have significant RPA binding. Here, too, there was a synergy between the two sub-domains in binding to RPA rather than a simple summation of binding by each of the sub-domains (data not shown). It is unlikely that in two separate instances the absence of RPA binding by the sub-domains is due to the RPA binding site spanning the site of division and thus being disrupted in each sub-domain. The alternative explanation is that weak RPA binding sites in each of the sub-domains synergize to produce the strong binding activity of the corresponding domain and this explanation is supported by additional data.

Aromatic amino acids in a sub-domain of p53 are important for RPA binding

The transcriptional trans-activator VP16 has been shown to interact with RPA, and a phenylalanine to proline mutation in VP16 shown to diminish RPA binding (He *et al.*, 1993; Li *et al.*, 1993). Reasoning that a similar mechanism of interaction occurred between RPA and p53, point-mutations were made in p53 which changed two adjoining aromatic amino acids, tryptophan and phenylalanine (residues 53–54) in one of the sub-domains of N2 to serines (W53S-F54S). This GST fusion protein did not bind RPA (data not shown). Several other point mutations have also been made in the N terminal part of p53 in the laboratory of Dr A Levine (Lin *et al.*, 1994), and a representative collection of these and W53S-F54S were engineered into GST-p53 fusion proteins and their RPA binding activity determined (Figure 2). The

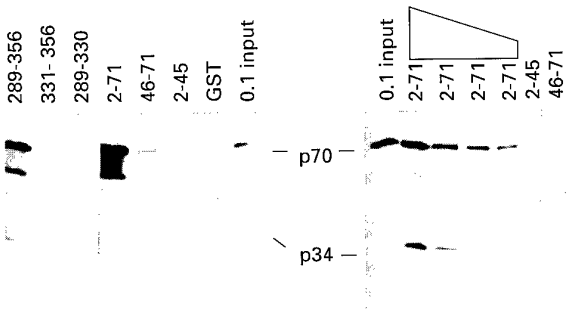

Figure 1 Sub-domains of p53 synergize to give strong RPA binding. (**a**) RPA bound to GST fusion proteins containing various p53 fragments (amino acids) 2–45, 46–71 and 2–71 in the N terminus; and amino acids 289–330, 331–356 and 289–356 in the C terminus) was detected by immunoblotting with anti-p70 and anti-p34 (RPA subunits) monoclonal antibodies. The anti-p34 antibody has a weaker titer than the anti-p70 antibody, so that at low RPA quantities, only p70 is visible. (**b**) Immunoblot probed with anti-p70 and anti-p34 showing titration of GST-p53 2–71, including levels of 100, 50, 30 and 10 percent the levels of both 2–45 and 46–71 sub-domains. Ten times as much of each sub-domain bound less RPA than the whole 2–71 domain. 0.1 input = one-tenth of input incubated with each of the GST fusion proteins. GST = negative control: no p53 fusion protein

results demonstrate that the aromatic residues W53 and F54 are important for RPA binding. Mutations in amino acids 48–49 (D48H-D49H) also decreased RPA binding, suggesting that negatively charged amino acids near the hydrophobic residues at 53–54 were important for RPA binding. The mutations which changed amino acids 22–23 of p53 (L22Q-W23S) affect its ability to activate transcription (Lin *et al.*, 1994), but did not affect its ability to bind RPA. Thus although in the herpesvirus transcriptional activator VP16 the same amino acid (F442) is important for both interaction with RPA and activation of transcription, this is not the case with p53. Therefore it seemed likely that we could separate the trans-activation and RPA binding functions of p53 with appropriate point-mutations.

Quantitation of RPA binding by increasing quantities of GST-p53 W53S-F54S vs GST-wild type p53 showed that the mutant was at least 10-fold weaker than wild type p53 at binding to RPA (Figure 3).

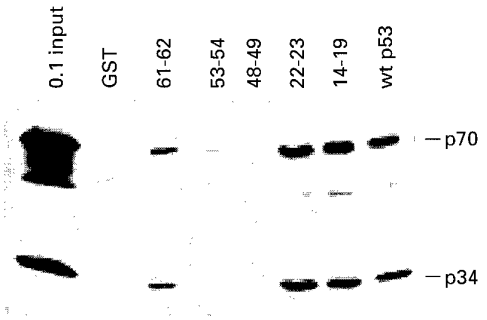

Figure 2 p53 mutants and RPA binding. RPA bound to GST fusion proteins containing p53 wild type (w.t.) and mutants were detected by immunoblotting with anti-p70 and anti-p34 (RPA subunits) monoclonal antibodies. The numbers above refer to the position of amino acids mutated from the original p53 wild type sequence as follows: L14Q-F19S; L22Q-W23S; D48H-D49H; W53S-F54S; and D61H-E62K. 0.1 = one-tenth S100 extract input. The GST = negative control: no p53 fusion protein

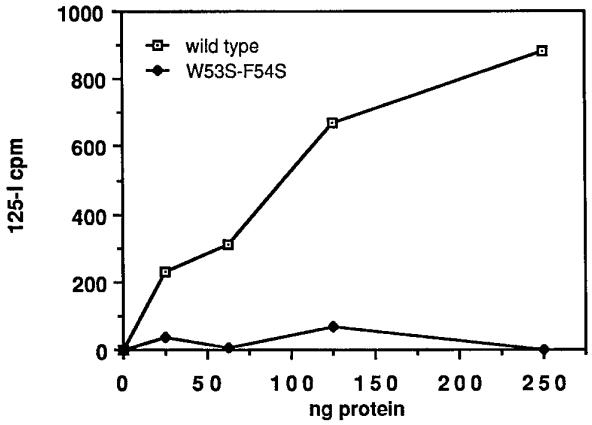

Figure 3 Titration of GST p53 wild type and W53S-F54S mutant proteins and RPA binding. Increasing amounts of GST-p53 or GST-W53S-F54S mutant proteins were added to 135 μg S100 extract. RPA binding was detected with immunoblotting with monoclonal anti-RPA 70 and 34 subunits, followed by ^{125}I-labeled rabbit anti-mouse antibody. ^{125}I-labeled bands were excised and counted in a gamma counter

A multimeric peptide containing aromatic and charged residues binds RPA

If a weak RPA binding site has aromatic and negatively charged residues, and if synergy between weak binding sites contribute to strong RPA binding we would predict that multimerization of a putative weak binding site would create a polypeptide that binds RPA well. Multimers of a twelve amino acid peptide surrounding the W53-F54 of p53 were fused to a GST protein and their ability to bind RPA tested (Figure 4). A monomer or even a dimer of the peptide bound RPA poorly, but a trimer and higher order multimers interacted with RPA strongly. A similar multimer of a peptide but with the critical tryptophan and phenylalanine changed to serines did not bind RPA.

To examine if this was a general mechanism by which other trans-activators also bound RPA, multimers of peptides from VP16 were tested for RPA binding (Figure 5 and Table 1). A list of all the peptides which possessed RPA binding activity (Table 1) supports the general rule that aromatic amino acids surrounded by negatively charged residues contribute to RPA binding. The variation in the lengths of the peptides which can bind RPA upon multimerization suggest that most of these multimers are not in a rigid structure like an alpha helix or beta sheet because such a structure would put constraints on the lengths of the repeating units that produced a functional RPA binding polypeptide.

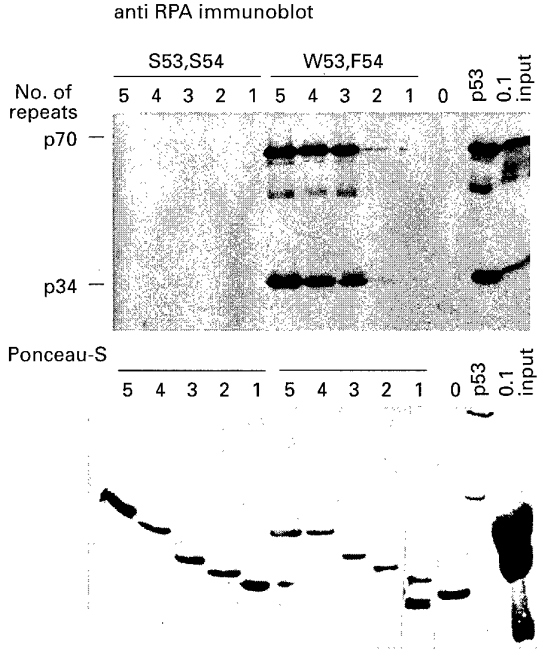

Figure 4 GST fusion proteins containing multimers of peptides from p53 wild type (W53,F54) and mutant (S53,S54) and the ability to bind to RPA. RPA bound to GST fusion proteins was detected by immunoblotting with anti-p70 and anti-p34 (RPA subunits) monoclonal antibodies. Ponceau S staining of the blot (bottom) demonstrates equal amounts of GST fusion protein loading in each lane. The carrier protein, casein, can also be seen in the input lane. p53 = GST p53; 0 = GST; 1, 2, 3, 4, 5 = number of repeats; 0.1 = one-tenth RPA input

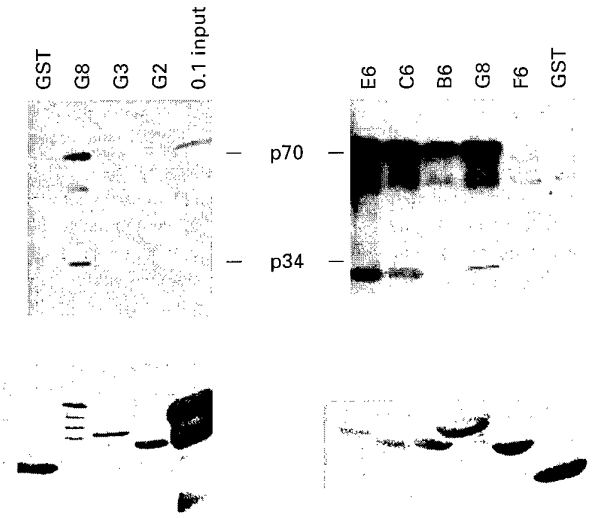

Figure 5 Ability of GST fusion proteins containing multimers of peptides derived from VP16 to bind to RPA. The sequences and amino acid position of the VP16 peptides that were multimerized (E,C,B,G,F) are in Table 1. The number after each letter indicates the number of repeats of each peptide (6 for E,C,B, and F; and 2,3, and 8 for G). RPA bound to these GST fusion proteins was detected as noted in Figure 1. Ponceau S staining of the blot (bottom) demonstrates equal amounts of GST fusion protein loading in each lane. GST = negative control; 0.1 input = one-tenth RPA input

Table 1 p53 and VP16 peptides binding to RPA

	Peptide	*RPA binding*
	48 58	
p53:	WF: DDIEQWFTEDG	+
	48 58	
	SS: DDIEQSSTEDG	–
	469 476	
VP16:	E: DMADFEFE	+
	437 445	
	B: DALDDFDLD	+
	437 448	
	G: DALDDFDLDMLG	+
	441 448	
	C: DFDLDMLG	+
	422 428	
	F: DELHLDG	–

The peptide sequence shown is the monomer that has been multimerized for RPA binding experiments (see methods). The sequences are derived from VP16 (E, B, G, C, F) and p53 wild type (WF) and mutant (SS). The numbers above each indicate amino acid number in the original protein. F is common in all the peptides that bind RPA

RPA from crude cell extracts does not bind to the 5C domain of p53

The W53S-F54S mutation in p53 produced a significant decrease in the binding of RPA from crude cell extracts (S100 from 293 cells) (Figure 2), even though the C terminal 5C domain of p53 had also been shown to interact with purified RPA (Dutta *et al.*, 1993). One explanation could be that 5C is unable to bind RPA from cell extracts. When tested directly, we found that while N2 could bind RPA from both purified fractions and from cell extracts, 5C could only bind RPA from the former (Figure 6). This effect was confirmed in cell extracts from SaOs2, H1299 and WRL68 (human embryo liver) cells. The above observation explains how we obtained a mutant form of p53 which loses the ability to bind RPA from cell extracts by making mutations only in the N2 (amino acids 2–121) domain of p53, leaving intact the dimerization and nuclear localization functions in the 5C domain which are essential for growth suppression.

Transcription activation by p53 mutants

To test the transcription activation properties of these p53 molecules, a transient transfection assay was done (Figure 7). Plasmids expressing p53 and various mutant derivatives were co-transfected into SaOs2 and H1299 cells (which lack endogenous p53) with a reporter plasmid, 6FSVCAT, which has six consensus p53 binding sites cloned upstream from a CAT gene (Unger *et al.*, 1993). Transcription activation by p53 was lowest in the L22Q-W23S mutant of p53 (approximately 25% of wild type), although it still retained fivefold (in SaOs2) and threefold (in H1299) activation over vector control. A defect in the transcription activation function of this mutant was first reported by Lin *et al.* (1994) where activity was comparable to that of vector alone. L14Q-F19S, D48H-D49H and D61H-E62K mutant forms of p53 retained 45–70% of transcriptional activity, comparable to activities also reported by Lin *et al.* (1994). The D48H-D49H and W53S-F54S versions of p53 possessed at least 45% wild-type trans-activation levels but significantly diminished RPA binding activities. Transcription activity of these mutants was confirmed by co-transfecting H1299 cells with another reporter plasmid, cosX1CAT, which contains the p53-responsive promoter of the mdm2 gene directing the expression of CAT (Lin *et al.*, 1994).

Plasmids expressing p53 mutants were transiently transfected into SaOs2 and H1299 cells, and cell lysates immunoblotted by Western analysis with anti-p53 antibody (1801, Oncogene) to ensure that protein expression of p53 mutants was comparable to that of

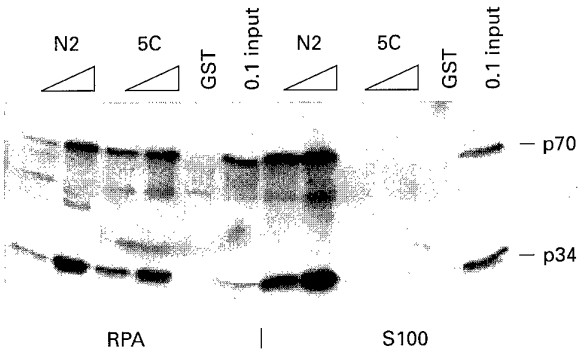

Figure 6 p53 5-C does not bind to RPA in crude cell lysates. RPA bound to GST fusion proteins p53 N-2 (amino acids 2–121) and p53 5-C (amino acids 289–393) were detected by immunoblotting with anti-p70 and anti-p34 (RPA subunits) monoclonal antibodies. GST = negative control; 0.1 = one-tenth input, which was pure RPA (left) and 293 cell extracts (S100) (right)

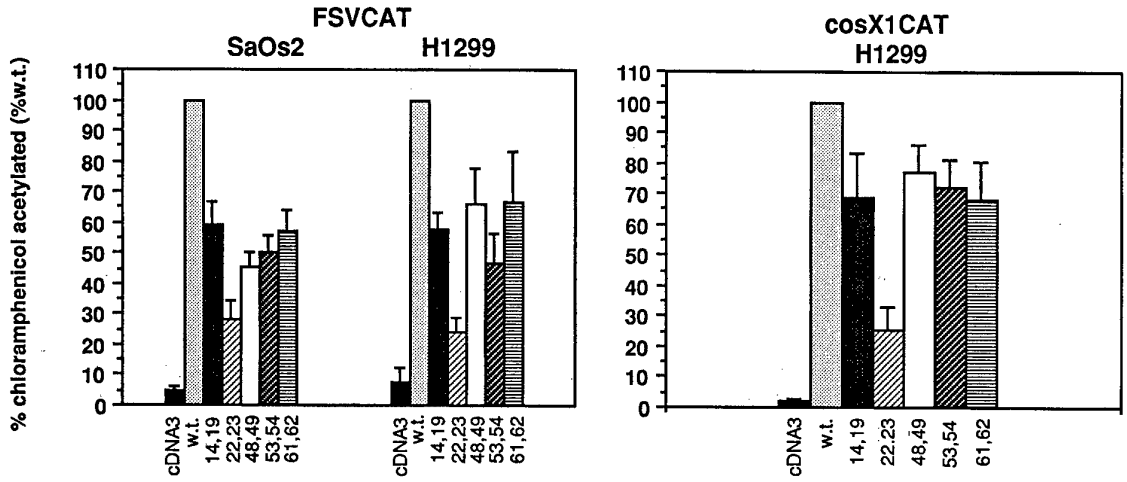

Figure 7 6FSV-CAT activity of p53 wild type and mutants transiently transfected into SaOs2 and H1299 cells (left) and cosX1-CAT activity in H1299 cells (right). CAT activity represents percent ^{14}C-chloramphenicol acetylated compared to p53 wild type (=100% activity) and represents the mean±SEM activity of eight plates from each mutant

wild type (data not shown). The same result was obtained by estimating the levels of p53 expression by immunofluorescence with anti-p53 antibodies. Because the D48H-D49H epitope was not recognized by the monoclonal antibody 1801, expression of this mutant was determined with the DO-1 antibody (Santa Cruz Biotechnology).

Transcription repression by p53 mutants

p53 represses transcription from TATA box containing promoters that do not have p53 binding sites, and the region of p53 responsible for this activity mapped to approximately the same region responsible for transcription activation (Lin *et al*., 1994). Transcription from the immediate early promoter of cytomegalovirus is repressed by p53 (Crook *et al*., 1994; Subler *et al*., 1994). We used the battery of mutant p53 expressing plasmids to determine how the mutations affect transcription repression (Figure 8). L14Q-F19S, D48H-D49H, W53S-F54S and D61H-E62K mutants of p53, which retained most of the trans-activation function, retained at least 50% of their trans-repression function. The L14Q-F19S mutant retained 40% of this function in H1299 and SaOs2 cells. L22Q-W23S, the mutant which was most reduced in transcription activation, was also the most impaired in transcription repression and was comparable to leaving out the plasmid expressing p53 (cDNA3 alone=100% loss of repression). This result suggests that the same residues of p53 involved in trans-activation and in contacting the basal transcription apparatus are also important for trans-repression.

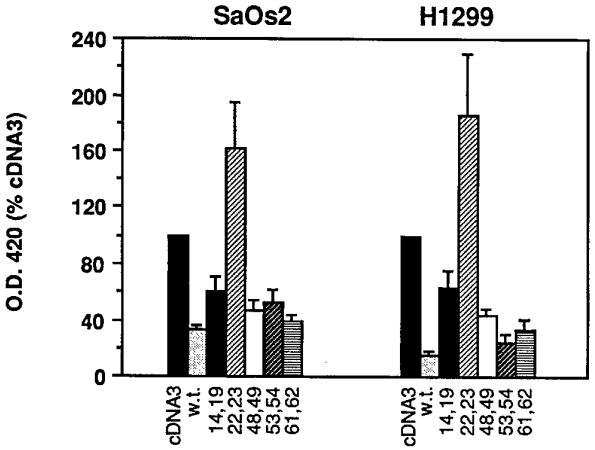

Figure 8 Beta-galactosidase activity of p53 wild type and mutants transiently transfected into SaOs2 and H1299 cells. Beta-galactosidase activity represents the O.D. 420 after addition of ONPG substrate compared to vector cDNA3 alone (=100%, no transcription repression) and represents the mean±SEM activity of eight plates from each mutant

Growth suppression by p53 mutants

Plasmids expressing wild type or mutant p53 were transfected into SaOs2 and H1299 cells (deficient in endogenous p53) and G418 resistant colonies selected (Figure 9). As demonstrated by others, plasmids expressing wild type p53 established very few G418 resistant colonies compared to the vector which does not express p53, due to growth suppression by p53. The results from the other plasmids demonstrate that both p53 proteins with wild type transcription trans-activation but diminished RPA binding, D48H-D49H and W53S-F54S, exhibited as much growth suppression as wild type p53 proteins. Therefore, both forms of p53 with diminished RPA binding retained growth suppression.

The p53 protein L22Q-W23S, which had wild type RPA binding activity, reduced transcription trans-activation, as well as transcription repression, showed diminished growth suppression in both SaOs2 and H1299 cells. The L14Q-F19S and D61H-E62K mutants, which retained most of the trans-activation and repression functions, also retained most of the growth suppression activity of wild type p53 in both

SaOs2 and H1299 cells. These results imply the trans-activation and/or repression properties of p53 are important for growth suppression.

To ensure that we did not miss a subtle effect of the W53S-F54S mutation on the ability of p53 to suppress entry into S phase, a transient transfection assay was done. Plasmids expressing p53 proteins were introduced into SaOs2 or H1299 cells and entry into S phase measured by BrdU incorporation as described by Delsal *et al.* (1995). BrdU was fluorescently detected by phycoerythrin-conjugated anti-mouse IgG2a (subclass of anti-BrdU primary antibody). p53 expression was detected by FITC-conjugated rabbit anti-mouse IgG1 (subclass of anti-p53 (1801) primary antibody). We found that the W53S-F54S mutant had slightly decreased ability to stop entry of cells into S phase. However, several other mutants of p53 (which retain RPA binding) were equally diminished (data not shown). Therefore, we believe that the RPA binding activity of p53 is not critical for growth suppression.

Discussion

Relatively short peptides with aromatic amino acids surrounded by negatively charged residues produce RPA binding activity when repeated several times in a protein. The degenerate nature of this RPA binding signal probably accounts for a number of proteins reported to bind RPA. Since similar bulky hydrophobic residues in the context of negatively charged amino acids are also essential for transcriptional trans-activation (Cress *et al.*, 1991), this observation may explain why several transcriptional activators are able to bind RPA. Potentially, this could be a common feature of several protein-protein interactions. The recently reported interaction between the DNA repair protein XP-G and RPA also uses a negatively charged part of XP-G (He *et al.*, 1995), and it is possible that the same mode of interaction is involved. The creation of a potent RPA binding site by multimerization of a slightly degenerate peptide module is novel. We suspect that several biologically relevant low affinity interactions (such as those cited above and those between transcriptional activators and the basal transcription apparatus) could be mediated by similar interaction surfaces.

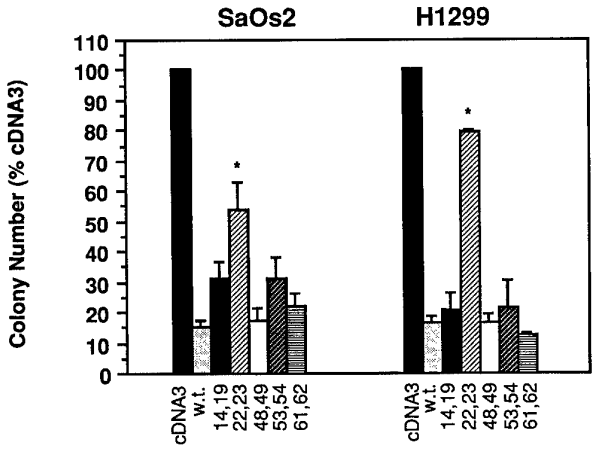

Figure 9 Growth suppression of wild type p53 and mutants in stable transfection assays. Bars represent the mean ± SEM of the number of colonies for 14 (SaOs2) and 4–5 transfections (H1299) compared to cDNA3 (=100%, no growth suppression). Data were analysed by one-way ANOVA and means were categorized by Fisher's LSD test. *indicates a significant difference compared to all other p53 alleles at $P<0.0003$ (SaOs2) and $P<0.0001$ (H1299)

Table 2 Summary of results

p53	*RPA binding*	*Transcription activation*	*Transcription repression*	*Growth suppression*
wild type	+	+	+	+
L14Q-F19S	+	+	+	+
L22Q-W23S	+	–	–	–
D48H-D49H	–	+	+	+
W53S-F54S	–	+	+	+
D61H-E62K	+	+	+	+

Bulky hydrophobic residues in the N terminal region of p53 have already been implicated in transcriptional trans-activation and in interactions with the TATA binding protein and viral and cellular oncogenes E1b and mdm2 respectively (Lin *et al.*, 1994). Although our results suggest that at least for RPA binding, the interaction depends on relatively degenerate sequences, there must be some specificity to the modules that interact with transcriptional factors vs the ones that interact with RPA because we were able to create point mutations which affected RPA binding but not trans-activation and vice-versa. The exact source of this specificity will become more clear when the interaction domains of the other partners (RPA, TAFII40, E1b or mdm2) in these interactions are defined.

Peptides of varying lengths could be multimerized to produce RPA binding. Therefore, there do not appear to be strict structural constraints on the interactor modules, because each of the multimers have different distances between the aromatic residues, between the acidic residues and between the aromatic and acidic residues. We think that the interactor modules are unstructured, and may be induced into a more defined structure when the other partner is bound. Recent biophysical assays indeed demonstrated that the transcriptional activation domain of VP16 is relatively unstructured in the free state and acquires some structure when complexed to the basal transcriptional apparatus (Shen *et al.*, 1996 a,b). This 'induced fit' hypothesis also leaves room for specificity of interaction depending on the structure of the other partner in the interaction. The minimal requirements of the other partner could be to have a distribution of bulky hydrophobic residues surrounded by positively charged residues, so that hydrophobic and electrostatic interactions would stabilize the interaction.

We do not yet know why the 5C region did not bind to RPA from the crude cell extracts. Either the RPA from cell extracts is present in a form where it is not able to interact with 5C, or the cell extract contains factors which bind to 5C and prevent RPA from binding. The effect is probably not due to pre-existing p53-RPA complexes, since two cell lines in which the effect was shown, SaOs2 and H1299, lack functional alleles of p53. The TATA box binding protein (TBP) binds to the 5C region (Horikoshi *et al.*, 1995).

Potentially, TBP or similar factor(s) could bind to 5C in cell extracts and prevent RPA from binding to the same.

L22Q-W23S showed decreased transcription activation, loss of transcription repression, wild type RPA binding and decreased growth suppression, indicating that transcriptional trans-activation and/or transcription repression is most important for growth suppression. RPA binding, in contrast, lost in the D48H-D49H and W53S-F54S alleles of p53, appears unimportant for growth suppression.

As mentioned in the introduction, p53 has other functions relevant to the production of cancers. It is required to induce apoptosis in response to x-irradiation or chemotherapy, to produce a pause in DNA replication after a sub-lethal dose of radiation so as to give the cell time to repair its DNA, and to prevent gene amplification. p53 can induce apoptosis through a pathway independent of new mRNA transcription and protein synthesis (Caelles *et al.*, 1994), making it likely that the transcriptional trans-activation function of p53 is occasionally dispensable for this activity. p53 has recently been shown to selectively bind to insertion-deletion mismatch lesions (Lee *et al.*, 1995), and by analogy with the XP-A-RPA interaction (He *et al.*, 1995), may be involved in recruiting RPA to these sites of DNA repair. The p53-RPA interaction could be important for apoptosis induction and/or the other functions of p53 described in this paragraph. Future experiments will be directed toward testing these possibilities.

An examination of which p53 mutants retain or lose trans-repression suggests possible mechanisms for the trans-repression. Hydrophobic residues at 22–23 important for trans-activation are also required for trans-repression so that sequestration of basal transcription factors by p53 is likely to be important for trans-repression. The sequestration could be executed through the low specificity interactor modules containing hydrophobic residues as described for the RPA-p53 interaction. Data from other groups has suggested that the oligomerization domain at the C terminus of p53 is important for trans-repression activity. (Subler *et al.*, 1994). Therefore trans-repression probably involves multimers of p53 sequestering basal transcription factors in complexes away from functional promotors.

In conclusion we have determined which feature of p53 and other trans-activators promotes interaction with RPA, and shown with two mutant alleles of p53 that growth suppression occurs independent of binding to RPA. We have also shown that trans-repression by p53 is affected by mutations at residues 22–23 (which are also important for trans-activation), and this allele is most defective in growth suppression. These results suggest that while RPA-p53 interaction is not important, transcription trans-activation and/or trans-repression by p53 are important for cell growth suppression.

Materials and methods

p53 wild type and mutant constructs

p53 fragments (amino acids 2–71; 2–45; 46–71; 1–121; 289–393; 289–330; 331–356) were generated by PCR with appropriate pairs of oligonucleotides as primers using a clone of p53 cDNA as a template. Fragments were then cloned into BamHI and Asp 718 sites of pet11GTK vector (Dutta *et al.*, 1993), such that the GST protein reading frame was fused in frame with the p53 fragment. To make the W53S-F54S allele of p53, site-directed mutagenesis was performed by both a PCR based method and the Kunkel method (Sambrook *et al.*, 1989). Mutant alleles were sequenced to ensure that no additional mutations were created.

Peptide multimers

Oligonucleotides corresponding to p53 amino acids 48–58 (both wild type and W53S, F54S mutants) were synthesized. Partial BglII and BamHI sites were generated at the ends of the oligos to facilitate the oligomerization. Corresponding oligonucleotides were phosphorylated, annealed and ligated to BamHI site of pet11GTK. Clones containing insertions were screened by colony PCR. All clones containing different numbers of insertions were rechecked by PCR and confirmed by sequencing. The GST fusion proteins containing multimers of peptides from VP16 will be described elsewhere (M Tanaka, unpublished observations).

RPA-p53 interaction assay

The GST-p53 fusion proteins were produced and purified on glutathione agarose beads as described previously (Dutta *et al.*, 1993). Beads carrying 400–600 ng GST and 200–300 ng GST p53 fusion proteins were used in the assays, and incubated with either 125 ng pure RPA or 135 μg S100 extract from 293 cells (transformed primary embryonal kidney, human) as indicated. RPA purification from human 293 cell extracts, and the assay for binding of RPA by GST-p53 fusion proteins has also been described (Dutta *et al.*, 1993). For the accurate quantification of RPA binding shown in Figure 3, the Westerns were developed with ^{125}I labeled rabbit anti-mouse IgG (Dupont Chemicals). ^{125}I labeled bands were excised and counted in a gamma counter.

Growth suppression by stable transfections

CMV/p53 mutants 14–19, 22–23, 48–49 and 61–62 were the kind gift of Dr Arnold Levine. p53 wild type and W53S-F54S mutants were cloned into a mammalian expression vector cDNA3 (Invitrogen) which expresses genes inserted downstream from a cytomegalovirus (CMV) promoter and which contains a neomycin phosphotransferase gene and an SV40 origin of DNA replication. These plasmids were transfected into SaOs2, a human osteosarcoma cell line with loss of both alleles of p53, as well as H1299, a human lung large cell carcinoma cell line with partial homozygous deletion of the p53 gene, by the calcium phosphate method. Exponentially growing cultures were transfected with 10 μg of each plasmid. After 24 h, cells were washed in phosphate buffered saline and fresh DMEM medium containing 10% fetal calf serum and G418 was added. The ability of each plasmid to produce G418 resistant colonies was measured as described (Chen *et al.*, 1995).

Transcription activation and repression

SaOs2 or H1299 cells were transfected with 10 μg of plasmids expressing p53 alleles (based on the cDNA3 vector), 5 μg of a reporter plasmid, 6FSVCAT, expressing the chloramphenicol acetyl transferase (CAT) gene downstream from a p53 consensus binding sequence containing six copies of the p53-binding element TGCCT (Unger *et al.*, 1993). Transcription activity was confirmed in H1299

cells by transfecting as above with a cosX1CAT plasmid containing a p53-responsive promoter from murine mdm-2 gene (Wu *et al.*, 1993). Activity in SaOs2 cells was not tested with this construct due to a high level of background CAT activity. Transcription repression was tested by transfecting as above with a reporter plasmid expressing the beta-galactosidase gene from a cytomegalovirus promoter known to be repressed by wild type p53 (Crook *et al.*, 1994; Subler *et al.*, 1994). Eight hours later, plates were washed twice in PBS and fresh medium (DMEM with 10% FCS) was added. After 36 h cells were harvested and lysed. Equal fractions of cell lysates from each of the transfected plates were assayed for CAT (transcription activation) and beta-galactosidase (transcription repression) activity (Sambrook *et al.*, 1989). CAT and beta-galactosidase activity were expressed as percentage of activity relative to plates with wild type (100%) and cDNA3 vector alone (100%), respectively.

Statistics

Data for growth suppression assays were statistically analysed by one-way analysis of variance, and means were categorized by Fisher's LSD test (Steel *et al.*, 1980).

Acknowledgements

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A 39 amino acid fragment of the cell cycle regulator p21 is sufficient to bind PCNA and partially inhibit DNA replication *in vivo*

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ABSTRACT

The cell cycle regulator p21 interacts with and inhibits the DNA replication and repair factor proliferating cell nuclear antigen (PCNA). We have defined a 39 amino acid fragment of p21 which is sufficient to bind PCNA with high affinity (K_d 10–20 nM). This peptide can inhibit DNA replication *in vitro* and microinjection of a GST fusion protein containing this domain inhibited S phase *in vivo*. Despite its high affinity for PCNA, the free 39 amino acid peptide does not have a well-defined structure, as judged from circular dichroism and nuclear magnetic resonance measurements, suggesting an induced fit mechanism for the PCNA–p21 interaction. The association of the small peptide with PCNA was thermolabile, suggesting that portions of p21 adjoining the minimal region of contact stabilize the interaction. In addition, a domain containing 67 amino acids from the N-terminus of PCNA was defined as both necessary and sufficient for binding to p21.

INTRODUCTION

Normal cell cycle progression involves a sequential increase in the levels of various cyclins, their association with corresponding cyclin-dependent kinases (cdk) and sequential activation of these kinase activities in the different phases of the cycle. Cyclins, cdk kinases, the cdk inhibitor p21 and the DNA replication factor proliferating cell nuclear antigen (PCNA) have been found to form a quaternary complex in untransformed cells (1–3). Besides associating with and inhibiting cdk2 kinase (4–8), p21 has an additional activity through its interaction with the DNA replication factor PCNA. PCNA is an auxiliary factor for DNA polymerases δ and ε and is essential for DNA replication *in vitro* and *in vivo* (9–15). p21 interacts with PCNA and inhibits its activity (16–21). p21 is transcriptionally induced by the tumor suppressor protein p53, which is itself increased in response to DNA damage, and it has been suggested that the p21 is an important effector of the growth suppressive function of p53 (22).

We and others have reported that the N-terminal 90 amino acids of p21 inhibited cyclin–cdk kinase activity, DNA replication in *Xenopus* egg extracts and cell growth in *p53* null-transformed cancer cell lines (19,23–25). The C-terminal 77 amino acids of p21 interacted with PCNA and inhibited SV40-based DNA replication and *Xenopus* DNA replication. A small chemical based on the structure of p21 which interacts with and inhibits PCNA could be useful for suppressing cell growth or for inhibiting DNA repair after radio- or chemotherapy. We have determined that peptides based on the structure of p21 suppress cell growth when delivered *in vivo* at high concentrations and have measured the K_d of the peptide–PCNA interaction to determine if it was suitable for pharmacology. Despite the high affinity of the peptide–PCNA interaction, circular dochroism and NMR studies show that the free peptide is flexible in structure, suggesting an induced fit mechanism for the peptide–PCNA interaction. We also demonstrate that the N-terminal 67 residues of PCNA are necessary and sufficient for the interaction and that there are multiple potential binding sites for p21 on each PCNA trimer. Taken together these results indicate that while a peptide derived from p21 may itself be unsuitable for targeting the DNA replication and repair apparatus, a synthetic chemical based on the structure of the PCNA-bound peptide could be effective *in vivo*.

MATERIALS AND METHODS

Plasmids

pGST-p21, pGST-p21N and pGST-p21C were generated as described (23). pGST-p21M1 and pGST-p21C2 were generated by PCR with *Pfu* polymerase and cloned into *Bam*HI and *Sal*I sites of pGEX-5X3 (Pharmacia). pETPCNA has been described (26).

Protein expression and purification

Bacterially produced proteins were expressed in *Escherichia coli* BL21. Protein induction, cell lysis and affinity purification with

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glutathione–agarose beads were as described (27). *In vitro* transcription–translation reactions were as suggested by the manufacturer (Promega).

Synthesis of peptides

A 41 amino acid p21C2 peptide (consisting of the 39 C-terminal amino acids of p21 plus two Lys residues at the C-terminal end required for chemical synthesis) was synthesized at the Harvard Medical School Biopolymer Laboratory using a Millagen/Biosearch 9600 synthesizer. The peptide was purified using C18 reverse phase HPLC.

The sequences of peptides used were:
p21C2:QAEGSPGGPGDSQGRKRRQTSMTDFYHSKRR-LIFSKRKPKK;
CSH262:WNSGFESYGSSSYGGAGGYTQAPGGFGAPAPS-QAEKKSRAR;
CSH119:ADAQHAAPPKKKRKVEDPKDF.

Assays

Affinity chromatography on glutathione beads coated with various GST fusion proteins ('pull-down' assays) was as described (23,27), except that washes were with buffer A7.4 (20 mM Tris–HCl, pH 7.4, 1 mM EDTA, 0.01% NP-40, 10% glycerol, 25 mM NaCl). Unlabeled proteins were detected by immunoblotting with appropriate antibodies and ECL reactions. Proteins produced by *in vitro* transcription–translation were labeled with [^{35}S]methionine and visualized by fluorography.

The SV40 DNA replication reaction was performed as previously described (23,28). Aliquots of 180 ng pSV011 were replicated in a 30 µl reaction containing 100 ng T antigen and 50 µg S100 extract from cell cycle asynchronous 293 cells. Cell extracts and T antigen were pre-incubated on ice for 30 min with GST fusion proteins or peptides without plasmid DNA, then replication reactions were performed by mixing plasmid DNA and incubation at 37°C for 1 h.

Gel filtration

Protein or protein mixtures were incubated on ice for 15 min in A7.4 buffer before loading onto a 25 ml Superose 12 gel filtration column (Pharmacia). Proteins were eluted from the column at a flow rate of 0.4 ml/min. Fractions of 0.5 ml were collected, separated by 15% SDS–PAGE and stained with Coomasie blue to visualize the proteins PCNA (37 kDa), Fen1 (45 kDa) and p21C2 (4.2 kDa).

Scatchard analysis

Bacterially expressed human PCNA was purified as described (26) and labeled with ^{125}I using Bolton–Hunter reagent and following the manufacturer's instructions (Du Pont). Varying amounts of GST–p21C or GST–p21C2 (at least a 30-fold molar excess compared with PCNA) were incubated with a fixed amount of radiolabeled PCNA for 1 h at 4°C or for 15 min at 37°C in buffer A7.4. The GST proteins were recovered by binding to glutathione–agarose beads and the amount of bound PCNA estimated by counting in a gamma counter. All points on the Scatchard plots are the result of at least four different binding assays done on at least two separate days. Care was taken to subtract non-specific binding to GST beads.

The data was analyzed by Scatchard plot according to the equation

$$b/R_t = -b/K_d + B_{max}/K_d$$

where b is the concentration of bound PCNA (in c.p.m./200 µl), R_t is the total concentration of GST fusion protein (in nM), B_{max} is the concentration of total PCNA that can be bound by the GST fusion protein (c.p.m./200 µl) and K_d is the dissociation constant (in nM). K_d was estimated from the slope of the graph of b/R_t versus b (29).

Microinjection

IMR90 human diploid fibroblast monolayers growing on glass coverslips (at 60% density) were synchronized in G0 by serum starvation for 48 h and stimulated to enter G1 by addition of 10% fetal bovine serum. Fifteen hours after re-activation cells in G1 were microinjected with the indicated proteins using an automated microinjection system (AIS; Zeiss). All microinjection experiments were carried out in 3.5 cm Petri dishes containing 3 ml carbonate-free DMEM, in order to avoid a decrease in pH of the medium during the injection. Each cell was injected with protein or peptide (3.75 mg/ml in PBS) together with normal rabbit immunoglobulin (2.5 mg/ml) at a pressure between 50 and 150 hPa. The computer settings for injection were angle '45', speed '10' and time of injection '0.0 s', so as to deliver 0.01–0.05 pl liquid/nucleus. For more details of the microinjection procedure see Pepperkok (30).

DNA synthesis was monitored by incubating with BrdU (100 µM; Amersham) for 10–12 h after microinjection. Coverslips were then rinsed in PBS and fixed for 10 min in –20°C cold methanol/acetone (1:1) and washed again three times with PBS. Microinjected cells were detected by incubation for 1 h with biotinylated horse anti-rabbit IgG (diluted 1:50; Vector Laboratories), washed three times with PBS and incubated with Texas red-conjugated streptavidin (diluted 1:100; Vector Laboratories). Coverslips were subsequently incubated for 10 min with 1.5 M HCl, washed three times with PBS and then incubated for 1 h with a solution of mouse monoclonal anti-BrdU antibody plus nuclease (undiluted; Amersham), followed by a 30 min incubation with a 1:50 dilution of an anti-mouse FITC-conjugated antibody (Vector Laboratories).

All antibody reactions were carried out in a humidified chamber at room temperature and dilutions were made in DMEM containing 10% FCS. Counterstaining for DNA was performed by adding 1 µg/ml bisbenzimide (Hoechst 33258) to the final PBS wash. Immunofluorescence samples were directly mounted in Crystal/mount medium (Biomeda Corp.). Photographs were taken using a Plan-Neofluar 40× lens mounted on a Zeiss Axiophot Photomicroscope and a Color Video Printer Mavigraph on Sony UPC-3010 print paper.

In each experiment ~100 injected cells (and a corresponding number of non-injected cells) were counted. Per cent inhibition of BrdU incorporation was calculated as $[(N-I)/N] \times 100$, where N is percentage BrdU incorporation in non-injected cells and I is percentage BrdU incorporation in cells microinjected with antibodies. The obtained numerical value is independent of possible experimental variations in the number of BrdU-positive cells that had not been injected.

Circular dichroism

Spectra were obtained at a concentration of 22 µM (p21C2) in PBS, pH 7.0. A path length of 0.1 cm in an Aviv 62DS spectropolarimeter equipped with a temperature control unit was used. Spectra were obtained with a scan speed of 1 s at each wavelength. Mean residue

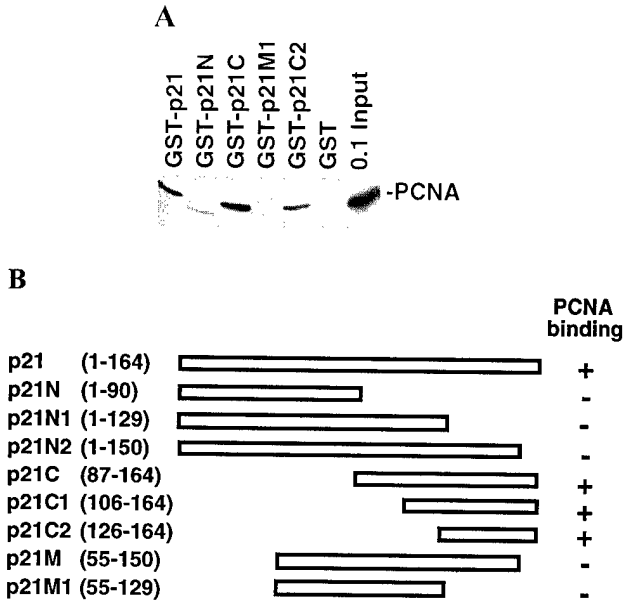

Figure 1. Deletion analysis of p21 shows that the C-terminal 39 amino acids are sufficient for binding PCNA. (**A**) Immunoblot with anti-PCNA antibody. The indicated GST fusion proteins were used to mediate the binding of bacterially produced human PCNA (37 kDa) to glutathione–agarose beads. One tenth of input PCNA is shown for comparison. The smaller band seen in the second lane is the GST–p21N protein, which is ~35 kDa in size. Due to their high protein content, GST fusion proteins produce background bands in the enhanced chemiluminescence reaction used to visualize the immunoblots. (**B**) Schematic summary of deletion derivatives of p21 and their ability to bind PCNA [(A) and data not shown]. The numbers indicate which amino acids of p21 are present in the deletion derivatives.

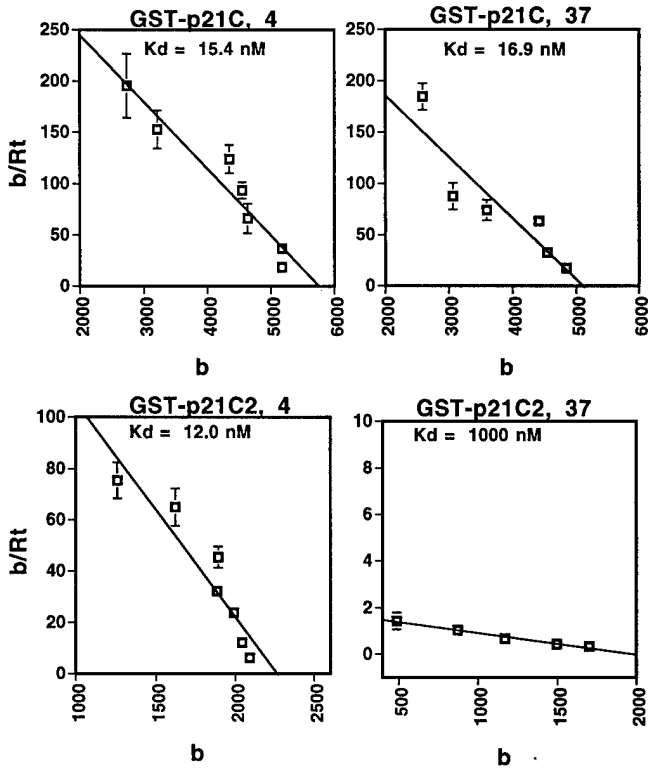

Figure 2. Scatchard analysis of the binding of PCNA to GST–p21C or GST–p21C2 at 4 or 37°C. The *y*-axis shows the ratio of bound PCNA (c.p.m./200 µl) to the total concentration of the GST fusion protein (nM) (b/R_t). The *x*-axis shows the amount of bound PCNA (c.p.m./200 µl) (*b*). Each point is the mean ± SD of four measurements and the slope of the line equals $-1/K_d$.

ellipticity (θ) was calculated with a calculated molecular weight of 4562 g/m.

NMR spectra

All experiments were run on a Varian VXR500 spectrometer. Spectra were recorded at 2 mM sample concentration in PBS, 10% D_2O, pH 7.0. NOESY spectra were recorded at 5 and 25°C with mixing times of 150 and 300 ms. TOCSY spectra were recorded at 25°C with mixing times of 50 and 75 ms.

RESULTS

The C-terminal 39 amino acids of p21 are sufficient to interact with PCNA

Bacterially expressed glutathione S-transferase–p21 (GST–p21), GST–p21C and GST–p21C2 were used as an affinity matrix to demonstrate that PCNA interacts with the last 39 amino acids of p21 (Fig. 1 and data now shown). Scatchard analysis of the interaction (at 4°C) showed that the K_d values for the GST–p21C–PCNA and GST–p21C2–PCNA interactions were 15.4 and 12.0 nM respectively (Fig. 2). At 37°C the K_d of the GST–p21C–PCNA interaction was unchanged, but that of GST–p21C2–PCNA increased 100-fold. A synthetic 41 amino acid peptide corresponding to p21C2 (plus two lysines at the C-terminus) was synthesized. In agreement with the K_d measurements, the synthetic peptide competitively inhibited binding of PCNA to GST–p21 at 4°C (Fig. 3a), but failed to compete with GST–p21 for binding to PCNA at 37°C (Fig. 3b).

These experiments demonstrate that the C-terminal 39 amino acids of p21 are sufficient to bind PCNA. However, an additional 38 amino acids (present in GST–p21C but not in GST–p21C2) stabilize the interaction and prevent loss of affinity as the temperature is increased to the physiological range.

Inhibition of the SV40 replication reaction

Since the interaction of p21 with PCNA inactivates its function as a DNA replication factor, we measured the abilities of the GST fusion proteins to inhibit the SV40-based DNA replication reaction (Fig. 4). The concentration required to obtain 50% inhibition of replication (IC_{50}) was 0.5–1 µM for GST–p21 or GST–p21C and 9 µM for GST–p21C2. The synthetic p21C2 peptide was slightly weaker than GST–p21C2 at inhibiting SV40 replication (IC_{50} 14 µM), but addition of 1% DMSO to the replication reaction improved inhibition by the p21C2 peptide ~2-fold (data not shown). The 10- to 20-fold weaker inhibitory activity of GST–p21C2 compared with GST–p21C could be consistent with its lower affinity for PCNA at 37°C. Inhibition of DNA replication by p21C2 was reversed by addition of excess PCNA (data not shown). We tested whether amino acids 87–125 of p21 (present in p21C, but not in p21C2) contributed to inhibition of SV40 DNA replication by interacting with and inhibiting a second replication factor. A fragment of p21 containing this region, GST–p21M1, was unable to bind PCNA (Fig. 1) or inhibit the DNA replication reaction (Fig. 4). These results suggest that amino acids 87–125 of p21 contribute to replication inhibition only

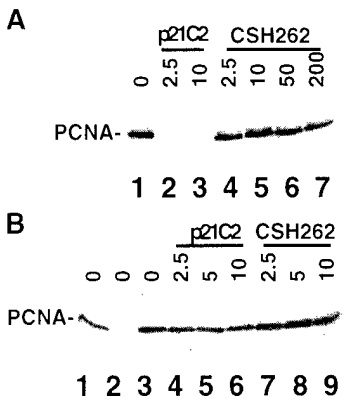

Figure 3. Synthetic p21C2 peptide can competitively inhibit p21–PCNA interaction at 4 but not at 37°C. Binding of PCNA visualized by immunoblotting of bead-bound proteins with anti-PCNA antibody. (**A**) 1 μM GST–p21 was incubated at 4°C with 100 μg S100 extract from 293 cells. Concentrations (μM) of peptide competitor are indicated at the top: p21C2 peptide (lanes 2 and 3) or negative control peptide CSH262 (lanes 4–7). (**B**) As (A) except the reaction was carried out at 37°C. Lane 1, one tenth input lysate; lane 2, bound to GST protein; lanes 3–9, bound to 1 μM GST–p21 protein. Competing peptides were none (lanes 1–3) or the indicated concentrations of p21C2 or negative control peptide CSH262.

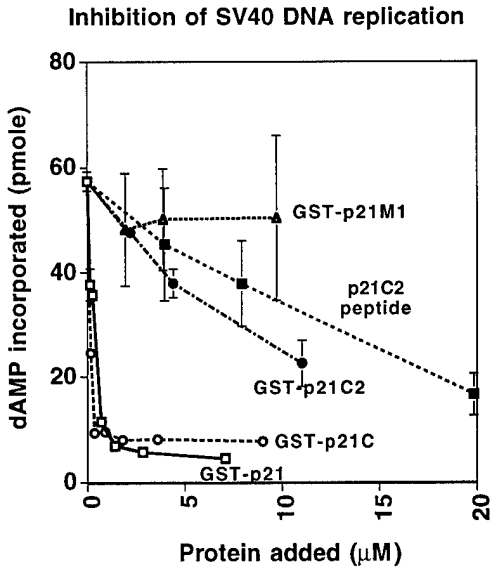

Figure 4. Inhibition of SV40 DNA replication by fragments of p21. The proteins added were GST–p21 (open squares), GST–p21C (open circles), GST–p21C2 (closed circles), GST–p21M1 (open triangles) and p21C2 peptide (closed squares). Each point represents the mean ± SD of three separate measurements of DNA replication (amount of dAMP incorporated into polynucleotide).

by stabilizing the p21–PCNA interaction. However, the 39 amino acid region of p21 was still an effective inhibitor of DNA replication *in vitro*.

Effect of GST–p21C and p21C2 peptides on entry of quiescent cells into S phase

To determine whether a p21-based peptide was active *in vivo* at reaching and interacting with PCNA we analyzed whether S phase was inhibited by these proteins. Quiescent diploid fibroblasts were stimulated by serum and entry into S phase followed after microinjection of GST fusion proteins or the p21C2 peptide (Fig. 5). GST–p21, GST–p21N and GST–p21C inhibited uptake of BrdU significantly compared with a negative control peptide CSH119, GST alone or GST fused to the cell cycle regulatory protein cdc25C (31). Thus GST–p21C inhibits growth of cells almost as well as GST–p21N when provided in high enough concentrations. Consistent with the result from the *in vitro* SV40 replication reaction, GST–p21C2 inhibited entry into S phase, although less effectively than GST–p21C. Surprisingly, the p21C2 peptide was only a weak inhibitor of cell growth. The difference between GST–p21C2 and the p21C2 peptide was observed consistently and was statistically significant ($P < 0.05$ by ANOVA). The results also confirm earlier reports that p21N, which binds and inhibits cdk kinases but not PCNA, inhibits growth of cells almost as effectively as p21.

Deletion mapping the part of PCNA which binds p21

Full-length PCNA and various deletion derivatives were synthesized by *in vitro* transcription–translation and binding to GST–p21 measured in a pull-down assay (Fig. 6). Since full-length PCNA bound to p21 well but a fragment of PCNA containing residues 40 to the C-terminus (40–C) did not, it appeared that the N-terminal portion of PCNA was important for binding p21. Consistent with this possibility, derivatives of PCNA containing amino acids 1–127 and 1–67 bound to p21. We conclude that a p21 binding domain of PCNA resides in the N-terminal 67 amino acids, perhaps even in the N-terminal 40 residues. The 1–127 fragment could have interacted with p21 indirectly as part of a larger complex with a protein present in the reticulocyte lysate (e.g. full-length rabbit PCNA). To test if this was the case the *in vitro* translation mix was fractionated by glycerol gradient sedimentation. The 'light' fractions, where the 1–127 fragment sediments in the same position as cytochrome c (and much lighter than the position of endogenous PCNA), could still associate with p21 (data not shown). Therefore, it is likely that the isolated 1–127 fragment of PCNA associates directly with p21.

The stoichiometry of the p21–PCNA interaction has been reported as 1:1 (p21 to trimer) (16) or 2.3:1 (18). Our observation that an isolated part of a PCNA monomer binds to p21 suggests that there could be more than one p21 binding site per PCNA trimer. p21C2 peptide was mixed with PCNA trimers at different ratios and subjected to gel filtration (Fig. 7). Even when p21C2 peptide was added at a ratio of 6 molecules peptide/PCNA trimer all the peptide was bound to PCNA and co-eluted with alcohol dehydrogenase (150 kDa). As a negative control p21C2 was mixed with another DNA replication/repair factor, Fen1, and subjected to gel filtration. All of the peptide eluted from the column after cytochrome c (14 kDa). The position of elution indicates that the p21C2 peptide is not present as a hexamer (30 kDa). Glycerol gradient sedimentation of 6-histidine-tagged p21 also indicates that the molecule exists as a monomer (16). Therefore, the association of virtually all the p21C2 peptide with PCNA even at a ratio of 6 peptide molecules/PCNA trimer is consistent with the model that there are multiple p21 binding sites per PCNA trimer (18). Even though there may be six potential binding sites for p21C2 peptide per PCNA trimer we favor a model where three molecules of p21 bind per PCNA trimer (see Discussion).

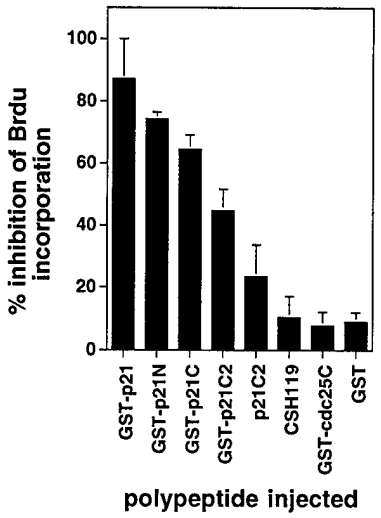

Figure 5. Inhibition of entry into S phase by microinjection of GST–p21 fusion proteins and indicated peptides into nuclei of serum re-activated diploid fibroblasts 15 h after re-activation. Mean ± SD for at least three different experiments are shown. CSH119, GST and GST–CDC25C were the negative controls, with indicated growth inhibition probably being a side effect of the injection procedure.

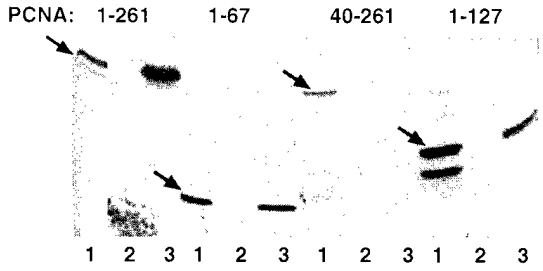

Figure 6. Deletion mapping of the part of PCNA that binds to p21. Full-length (1–261) or fragments containing the indicated residues of PCNA were produced by *in vitro* transcription–translation and visualized by fluorography. Lane 1, one tenth input; lane 2, protein bound to GST-coated beads; lane 3, protein bound to GST–p21-coated beads. Arrows indicate the PCNA (full-length or deletion derivative) in the input lane of each set.

Secondary structure of p21C2 peptide by circular dichroism and nuclear magnetic resonance

In view of the high affinity with which GST–p21C2 binds PCNA and inhibits S phase *in vivo*, a structural analog of the p21C2 domain would be a strong candidate for pharmacological use. Since the p21C2 peptide bound PCNA well at 4°C, we attempted to determine its structure.

The structure of the synthetic peptide p21C2 was studied by circular dichroism. A representative spectrum is shown in Figure 8. The peptide does not appear to have a well-defined secondary structure. The spectrum displays a minimum at 200 nm and a maximum at 220 nm (32). Temperature dependence of the CD spectra was monitored at 200 and 220 nm (Fig. 8, inset) and failed to show any appreciable change in ellipticity with change in temperature, confirming the lack of folded structure.

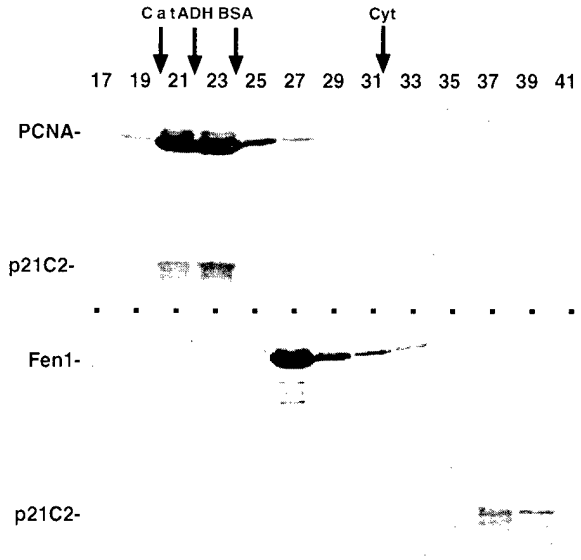

Figure 7. Gel filtration analysis of complexes formed by p21C2 peptide with PCNA (top) and Fen1 (bottom). Peptide and PCNA (molar ratio 2:1, equal to 6 molecules peptide/PCNA trimer) or peptide and Fen1 (molar ratio 2:1) were incubated and the complexes analyzed by chromatography on a Superose12 column. Alternate fractions were visualized by SDS–PAGE and Coomasie blue staining. The positions of elution of molecular weight markers is indicated by arrows at the top: catalase (Cat, 240 kDa), alcohol dehydrogenase (ADH, 150 kDa), bovine serum albumin (BSA, 66 kDa) and cytochrome c (Cyt, 12.5 kDa).

To further investigate the structure of the peptide ^{1}H NMR experiments were run in aqueous conditions. The NOESY spectrum (Fig. 9) lacks any significant number of inter-residue cross-peaks. The only cross-peaks seen are intra-residue, between amide protons and α protons (Fig. 9c) or side chain protons (Fig. 9d) of the same residue or sequentially adjacent residues. In particular, amide–amide cross-peaks are absent, which would be characteristic of an organized protein structure (Fig. 9a). Furthermore, the chemical shift values of each amino acid determined by TOCSY experiments are identical (within experimental error) to published random coil ^{1}H chemical shift values (data not shown) (33).

Altogether, the above spectroscopy results (circular dichroism and NMR), which did not vary under a large variety of aqueous conditions (temperature, buffer and pH; data not shown), demonstrate that unbound p21C2 does not adopt a well-defined structure in an aqueous environment.

DISCUSSION

The DNA replication enzymes are attractive targets for development of new agents for chemotherapy (34). We examined the p21–PCNA interaction with the long-term goal of determining if it could be exploited for the design of drugs which reach their target (PCNA) *in vivo*. As a first approximation we used a peptide (p21C2) derived from p21 which interacted with PCNA and inhibited the SV40 replication reaction *in vitro*. A 10-fold higher concentration of GST–p21C2 or the free p21C2 peptide was required to inhibit the SV40 replication reaction compared with GST–p21C. This is likely to be due to the 100-fold decrease in affinity of p21C2 for PCNA at physiological temperatures, although we cannot rule out the existence of factors in cell extracts that specifically interfere with the action of p21C2, but not p21C.

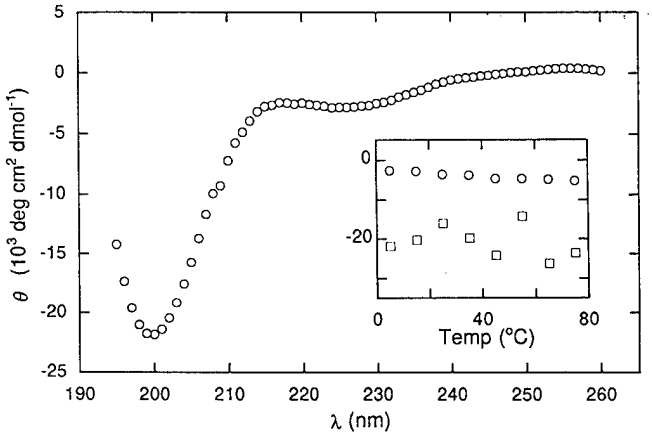

Figure 8. Far-UV circular dichroism spectrum of the p21C2 peptide in PBS (pH 7) at 4°C. (Inset) Effect of temperature on ellipticity measured at 220 nm (squares) and 200 nm (circles).

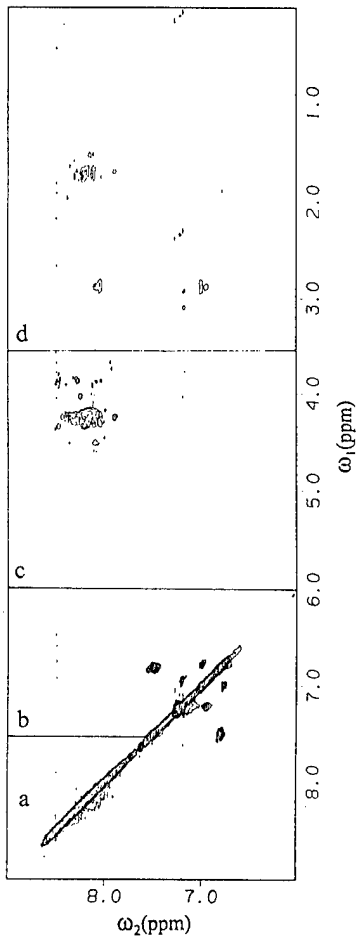

Figure 9. ^{1}H NOESY spectrum of p21C2 peptide in PBS at 25°C with a 150 ms mixing time. The low field half (ω_2 6.0–9.0 p.p.m.) of the spectrum is shown. Above the diagonal the spectrum is divided into four regions (a–d) where cross-peaks between distinct proton types occur (see text).

These results agree with a recent report that a 20 amino acid peptide from the C-terminal part of p21 (141–160) binds and inhibits PCNA *in vitro* (35). Point mutations have also indicated that multiple amino acids in this same region of p21 and additional ones at residues 161–163 are crucial for interaction with PCNA (36).

The efficacy of p21-based peptides at reaching and inhibiting PCNA *in vivo* was not clear before the present study. Because GST–p21C2 effectively inhibited cell growth but the free p21C2 peptide did not, we suspect that smaller peptides are unlikely to be useful in inhibiting PCNA *in vivo*. However, the high affinity of the interaction between GST–p21C and PCNA (K_d 10–20 nM) suggests that this interaction is suitable for pharmacological purposes. For comparison, other protein–protein interactions which have the potential for development as therapeutic agents include inhibition of cyclin–cdk kinases by p21 (K_i 1 nM) (37), interaction between phosphotyrosine-containing peptides and SH2 domains (K_d 10–100 nM) (38,39) and interaction between SH3 domains and proline-rich peptides (K_d 1000 nM) (40).

In general, peptide-based therapeutic agents suffer from the obvious problem of delivering peptides into cells at high concentrations. Our results point to two additional drawbacks: decreasing the length of the interacting peptide rendered the interaction thermodynamically unstable and additional poorly understood mechanisms were responsible for the small p21C2 peptide, but not GST–p21C2 protein, being inactivated in the cell. A small chemical that can mimic the structure of the active PCNA binding region of p21C2 may overcome all these drawbacks. Such a chemical may also be used to target other replication inhibitors to the site of DNA synthesis. Therefore, the best approach will be to determine the structure of the p21C2 binding interface and design chemicals which mimic this. Results reported in this paper indicate that the free p21C2 peptide lacks organized structure, suggesting that one has to determine the structure of the PCNA-bound peptide for this purpose.

Differences in the relative intracellular concentrations of p21C probably explain why S phase is inhibited by microinjection of GST–p21C, while transfection of plasmids expressing p21C failed to inhibit colony formation in an earlier assay (23,24). Expression from transfected plasmids is unlikely to yield as high a concentration of p21C per nucleus as is obtained by microinjection. Quiescent diploid fibroblasts have very little PCNA and as they enter the cell cycle new PCNA has to be synthesized to support DNA replication. Therefore, the low levels of PCNA and the fact that new PCNA is not sequestered in replication complexes are additional factors which favor cell growth suppression by p21C in the experiments reported here.

Using the two-hybrid method of studying protein–protein interactions another group has studied the domain of PCNA that interacts with p21 (35). A series of progressively increasing N-terminal deletions showed that amino acids 50–261 (50-C-terminus) and 100–261 of PCNA could interact with p21, but amino acids 150–261 could not, suggesting the importance of amino acids 100–150 of PCNA in the interaction with p21. Our biochemical method of assaying p21–PCNA interaction fails to show an interaction with the 40–261 derivative of PCNA, yet shows significant interaction of amino acids 1–67 or 1–127 of PCNA with p21. The two-hybrid method uses a version of PCNA with the yeast Gal4 activation domain fused at the N-terminus. Such a fusion may partially denature PCNA and permit interactions not possible with the trimeric PCNA complex. We find that GST–PCNA (where GST protein is fused to the N-terminus of PCNA) does not bind p21 (data not shown), so that the Gal4–PCNA fusion may also have inactivated the N-terminal p21 binding site. Alternatively, each PCNA molecule being composed of two structurally homologous

domains, amino acids 1–67 and 100–150 contain structurally similar N-terminal regions from each of the two domains (41). Therefore, the interaction with p21 could be executed by two structurally homologous regions of PCNA: a strong p21 binding region at the N-terminus and a weak binding region at amino acids 100–150. The weaker binding site in the 40–261 derivative may not be sufficient to give a positive signal in our assay, but could give a signal in the more sensitive two-hybrid assay. If this alternative is correct, each PCNA trimer may have up to six potential binding sites for p21.

The flexible nature of the p21C2 peptide may have been created by deletion of more than 75% of the p21 protein. However, despite the flexible structure, the high affinity and specificity of GST–p21C2 for PCNA at 4°C suggests that p21C2 is induced into a specific conformation when interacting with PCNA. This possibility is also favored by the observation that interaction of the 39 amino acid region is temperature sensitive and that the adjoining 38 amino acids stabilize the interaction (GST–p21C2 versus GST–p21C at 37°C).

Since isolated PCNA monomers and portions thereof bind p21, there are likely to be more than one p21 binding site per PCNA trimer. Are there six binding sites per PCNA trimer? We have provided experimental evidence which suggest that six p21C2 peptides could bind per PCNA trimer. However, it is unlikely that six molecules of p21 could bind to PCNA and not change the sedimentation profile of PCNA (16). Surface plasmon resonance spectroscopy showed that 2.3 molecules of p21 bind per PCNA trimer (18). Therefore, although there may be six sites per PCNA ring for association with p21, only a fraction of these can be occupied simultaneously by a molecule as large as p21. Nevertheless, multiple p21C2 binding sites on each PCNA ring translate into multiple binding sites for a chemical based on the binding interface of the peptide.

In conclusion, these results indicate that the p21–PCNA interaction has properties that may be useful for the design of drugs targeted to the replication fork. The affinity of the interaction is high, as are the number of binding sites per PCNA trimer. GST fusion proteins containing the peptide can interact with PCNA in the cell. However, direct use of peptides based on p21 will not be useful. Instead, one will have to determine the structure of the binding interface of p21 in the p21–PCNA complex and design chemicals based on this structure.

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